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APPLICATION  
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TITLE : THERAPEUTIC AND DIAGNOSTIC TOOLS FOR  
IMPAIRED GLUCOSE TOLERANCE CONDITIONS

THERAPEUTIC AND DIAGNOSTIC TOOLS  
FOR IMPAIRED GLUCOSE TOLERANCE CONDITIONS

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Cross Reference To Related Applications

This application is a continuation of application Serial Number 08/857,076,  
filed August 3, 2000, which is a continued prosecution application of Serial  
Number 08/857,076, filed May 15, 1997. <sup>now vs. pt. 6,225,120.</sup>

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<sup>4/11/03</sup>

Statement as to Federally Sponsored Research

This invention was made in part with Government funding, and the  
Government therefore has certain rights in the invention.

Background of the Invention

This invention relates to compositions and methods useful for delaying or  
ameliorating human diseases associated with glucose intolerance.

Diabetes is a major disease affecting over 16 million individuals in the  
United States alone at an annual cost of over 92 billion dollars.

Type I diabetes or insulin-dependent diabetes (IDDD) is an autoimmune  
disease. In the IDDM patient, the immune system attacks and destroys the insulin-  
producing beta cells in the pancreas. The central role of insulin in human  
metabolism is to aid in the transport of glucose into muscle cells and fat cells. The  
body's inability to produce insulin results in hyperglycemia, ketoacidosis, thirst,  
and weight loss. In addition, diabetics often suffer from chronic atherosclerosis  
and kidney and eyesight failure. A patient with IDDM requires daily injections of  
insulin to survive.

The most common form of diabetes is non-insulin dependent diabetes  
(NIDDM) or Type II diabetes. Type II diabetes is a heterogenous group of

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disorders in which hyperglycemia results from both impaired insulin secretory response to glucose and decreased insulin effectiveness (i.e., insulin resistance). Older people who are overweight are at particular risk for Type II diabetes. Genetic studies have suggested that, Type II diabetes is found in families and that the disease may be due to multiple genetic defects. In addition, the link between obesity and Type II diabetes is strong. Approximately 80 percent of Type II diabetics are obese. Weight loss and exercise can be effective to keep blood glucose levels normal, reducing the long-term complications of the disease.

At present there are few reliable methods for presymptomatic diagnosis of a genetic predisposition for diabetes or obesity. The search for genetic markers linked to diabetes and obesity has proven difficult, and this is especially true for Type II diabetes.

Treatments for diabetes emphasize control of blood glucose through blood glucose monitoring. The majority of patients take oral medications and/or insulin injections for appropriate control. Treatment of diabetes is generally chronic and lifelong, and treatments are generally not satisfactory over the long run. In addition, insulin treatment may become increasingly ineffective as the disease progresses. While insulin has been known for decades, and within the past decade, the receptors for insulin and aspects of its signaling pathway have been identified, the transcriptional output from these signaling pathways have not been characterized. In addition, the molecular basis of the obesity-induced insulin resistance is unknown.

#### Summary of the Invention

We have discovered that the *C. elegans* metabolic regulatory genes *daf-2* and *age-1* encode homologues of the mammalian insulin receptor/PI 3-kinase signaling pathway proteins, respectively. We have also discovered that the DAF-

16 forkhead protein represents the major transcriptional output of this insulin signaling pathway. For example, we have discovered that it is the dysregulation of the DAF-16 transcription factor in the absence of insulin signaling that leads to metabolic defects; inactivation of DAF-16 reverses the metabolic defects caused by lack of insulin signaling in *C. elegans*. Finally, we have found that the *C. elegans daf-7, daf-1, daf-4, daf-8, daf-14, and daf-3* genes encode neuroendocrine/target tissue TGF- $\beta$  type signal transduction molecules that genetically interact with the insulin signaling pathway. Similarly, we have shown that the metabolic defects caused by lack of neuroendocrine TGF- $\beta$  signals can be reversed by inactivation of the DAF-3 transcription factor.

Together, this evidence indicates that the DAF-16, DAF-3, DAF-8, and DAF-14 transcriptional outputs of these converging signaling pathways regulate metabolism. In addition, these discoveries also indicate that insulin and TGF- $\beta$ -like signals are integrated in humans to regulate metabolism, and that the homologues of other DAF proteins are likely to be defective or down regulated in human diabetic pedigrees as well as obesity induced diabetes. These results therefore indicate that the *C. elegans daf* genes are excellent candidate genes and proteins for human disease associated with glucose intolerance, e.g., diabetes, obesity, and atherosclerosis. Our findings indicate that the human homologues of these *daf* genes and proteins mediate insulin signaling in normal people and may be defective or mis-regulated in diabetics. Moreover, our findings indicate that there are at least two classes of type II diabetics: those with defects in the TGF- $\beta$  signaling genes, and those with defects in insulin signaling genes. Below we describe exemplary sequence and functional characteristics of the human homologues of the *daf* genes.

The discovery of converging DAF-7 and DAF-2 insulin-like signaling indicates that many cases of obesity-induced and genetically-induced diabetes (and

obesity) may be treated by administration of a human DAF-7 polypeptide.

The discovery that defects in the TGF- $\beta$  signaling pathway can be suppressed by decreases in DAF-3 pathway activity and that defects in the insulin pathway can be suppressed by decreases in DAF-16 activity highlight the utility of transcriptional regulatory DAF proteins in drug development; in particular, drugs that inhibit the activity of these proteins are useful for reversing the effects of obesity-induced or genetically-induced defects in DAF-7 TGF- $\beta$  type or insulin signaling.

In one aspect, the invention features a substantially pure preparation of a DAF-2 polypeptide, which can be derived from an animal (for example, a mammal, such as a human, or an invertebrate, such as *C. elegans*). In preferred embodiments, the DAF-2 polypeptide has insulin receptor (InR) activity, insulin receptor related activity, insulin-like growth factor receptor (IGF-1) receptor activity, or a combination of these activities.

The invention also features isolated DNA encoding a DAF-2 polypeptide. This isolated DNA can have a nucleotide sequence that includes, for example, the nucleotide sequence of the *daf-2* gene shown in Fig. 2B. This isolated DNA can also, in preferred embodiments, complement a *daf-2* mutation in *C. elegans*, an InR mutation in a mouse, or an IGF-1 receptor mutation in a mouse.

The isolated DNA encoding a DAF-2 polypeptide can be included in a vector, such as a vector that is capable of directing the expression of the protein encoded by the DNA in a vector-containing cell. The isolated DNA in the vector can be operatively linked to a promoter, for example, a promoter selected from the group consisting of *daf-2*, *age-1*, *daf-16*, *daf-1*, *daf-4*, *daf-3*, and *akt* promoters. The isolated DNA encoding a DAF-2 polypeptide, or a vector including this DNA, can be contained in a cell, such as a bacterial, mammalian, or nematode cell.

Also included in the invention is a method of producing a recombinant DAF-2 polypeptide, and a DAF-2 polypeptide produced by this method. This method involves (a) providing a cell transformed with isolated DNA that (i) encodes a DAF-2 polypeptide, and (ii) is positioned for expression in the cell, under conditions for expressing the isolated DNA, and (b) isolating the recombinant DAF-2 polypeptide.

A substantially pure antibody, such as a monoclonal or polyclonal antibody, that specifically recognizes and binds a DAF-2 polypeptide is also included in the invention.

The invention also features a method of detecting a gene, or a portion of a gene, that is found in a human cell and has sequence identity to the *daf-2* sequence of Fig. 2B. In this method, isolated DNA encoding a DAF-2 polypeptide, a portion of such DNA greater than about 12 residues in length, or a degenerate oligonucleotide corresponding to SEQ ID NOS: 33, 34, 79, 80, 81, 82, 83, or 84, is contacted with a preparation of DNA from the human cell under hybridization conditions that provide detection of DNA sequences having about 70% or greater nucleic acid sequence identity to the *daf-2* sequence of Fig. 2B. This method can also include a step of testing the gene, or portion thereof, for the ability to functionally complement a *C. elegans daf-2* mutant.

Another method included in the invention is a method of isolating a gene, or a portion of a gene, that is found in a human cell and has at least 90% nucleic acid sequence identity to a sequence encoding SEQ ID NOS: 33, 34, 79, 80, 81, 82, 83, or 84. This method involves (a) amplifying by PCR the human gene, or portion thereof, using oligonucleotide primers that (i) are each greater than about 12 residues in length, and (ii) each have regions of complementarity to opposite DNA strands in a region of the nucleotide sequence of Fig. 2B, and (b) isolating the human gene, or portion thereof. This method can also include a step of testing the

gene, or portion thereof, for the ability to functionally complement a *C. elegans* *daf-2* mutant.

In another aspect, the invention features a substantially pure preparation of a DAF-3 polypeptide, which can be derived from an animal (for example, a mammal, such as a human, or an invertebrate, such as *C. elegans*). In a preferred embodiment, the polypeptide is a SMAD protein. In other preferred embodiments, the polypeptide is capable of binding and interacting with a nematode DAF-1, DAF-4, DAF-8, DAF-14, or DAF-16 polypeptide.

The invention also features isolated DNA encoding a DAF-3 polypeptide. This isolated DNA can have a sequence that includes, for example, the nucleotide sequence of a *daf-3* gene shown in Figs. 11A, 11B, or 11C. This isolated DNA can also, in preferred embodiments, complement a *daf-3* mutation in *C. elegans* or complement a *daf-3* knockout mouse.

The isolated DNA encoding a DAF-3 polypeptide can be included in a vector, such as a vector that is capable of directing the expression of the protein encoded by the DNA in a vector-containing cell. The isolated DNA in the vector can be operatively linked to a promoter, for example, a promoter selected from the group consisting of *daf-3*, *daf-4*, *daf-16*, *daf-2*, *age-1*, and *akt* promoters. The isolated DNA encoding a DAF-3 polypeptide, or a vector including this DNA, can be contained in a cell, such as a bacterial, mammalian, or nematode cell.

Also included in the invention is a method of producing a recombinant DAF-3 polypeptide, and a DAF-3 polypeptide produced by this method. This method involves (a) providing a cell transformed with isolated DNA that (i) encodes a DAF-3 polypeptide, and (ii) is positioned for expression in the cell, under conditions for expressing the isolated DNA, and (b) isolating the recombinant DAF-3 polypeptide.

A substantially pure antibody, such as a monoclonal or polyclonal antibody, that specifically recognizes and binds a DAF-3 polypeptide is also included in the invention.

The invention also features a method of detecting a gene, or a portion of a gene, that is found in a human cell and has sequence identity to any of the *daf-3* sequences of Figs. 11A, 11B, or 11C. In this method, isolated DNA encoding a DAF-3 polypeptide, a portion of such DNA that is greater than about 12 residues in length, or a degenerate oligonucleotide corresponding to SEQ ID NOS: 35, 36, or 85, is contacted with a preparation of DNA from the human cell under hybridization conditions that provide detection of DNA sequences having about 70% or greater nucleic acid sequence identity to any of the *daf-3* sequences of Figs. 11A, 11B, or 11C. This method can also include a step of testing the gene, or portion thereof, for the ability to functionally complement a *C. elegans daf-3* mutant.

Another method included in the invention is a method of isolating a gene, or a portion thereof, that is found in a human cell and has at least 90% nucleic acid sequence identity to a sequence encoding SEQ ID NOS: 35, 36, or 85. This method includes (a) amplifying by PCR the human gene, or portion thereof, using oligonucleotide primers that (i) are each greater than about 12 residues in length, and (ii) each have regions of complementarity to opposite DNA strands in a region of any of the nucleotide sequences of Figs. 11A, 11B, or 11C, and (b) isolating the human gene, or portion thereof. This method can also include a step of testing the gene, or portion thereof, for the ability to functionally complement a *C. elegans daf-3* mutant.

In yet another aspect, the invention features a substantially pure preparation of DAF-16 polypeptide, which can be derived from an animal (for example, a mammal, such as a human, or an invertebrate, such as *C. elegans*). In a preferred



embodiment, the polypeptide is a forkhead transcription factor that binds DNA. In other preferred embodiments, the polypeptide is capable of interacting with a polypeptide selected from the group consisting of DAF-3, DAF-8, and DAF-14.

The invention also features isolated DNA encoding a DAF-16 polypeptide.

5 This isolated DNA can have a sequence that includes, for example, the sequence of the *daf-16* gene shown in Figs. 13A or 13B. This isolated DNA can also, in preferred embodiments, complement a *daf-16* mutation in *C. elegans*, or complement an FKHR or AFX mutation in a mouse.

10 The isolated DNA encoding a DAF-16 polypeptide can be included in a vector, such as a vector that is capable of directing the expression of the protein encoded by the DNA in a vector-containing cell. The isolated DNA in the vector can be operatively linked to a promoter, for example, a promoter selected from the group consisting of *daf-2*, *age-1*, *daf-16*, *daf-3*, *daf-4*, and *akt* promoters. The isolated DNA encoding a DAF-16 polypeptide, or a vector containing this DNA,  
15 can be contained in a cell, such as a bacterial, mammalian, or nematode cell.

Also included in the invention is a method for producing a recombinant DAF-16 polypeptide, and a DAF-16 polypeptide produced by this method. This method involves (a) providing a cell transformed with purified DNA that (i) encodes a DAF-16 polypeptide, and (ii) is positioned for expression in the cell,  
20 under conditions for expressing the isolated DNA, and (b) isolating the recombinant DAF-16 polypeptide.

A substantially pure antibody, such as a monoclonal or polyclonal antibody, that specifically recognizes and binds a DAF-16 polypeptide is also included in the invention.

25 The invention also features a method of detecting a gene, or a portion of a gene, that is found in a human cell and has sequence identity to the *daf-16* sequence of Figs. 13A or 13B. In this method, isolated DNA encoding a DAF-16

polypeptide, a portion of such DNA that is greater than about 12 residues in length, or a degenerate oligonucleotide corresponding to SEQ ID NO: 54, 55, 56, or 57, is contacted with a preparation of DNA from the human cell under hybridization conditions that provide detection of DNA sequences having about 70% or greater nucleic acid sequence identity to the *daf-16* sequence of Figs. 13A or 13B. This method can also include a step of testing the gene, or portion of the gene, for the ability to functionally complement a *C. elegans daf-16* mutant.

Another method included in the invention is a method of isolating a gene, or a portion of a gene, that is found in a human cell and has at least 90% nucleic acid sequence identity to a sequence encoding SEQ ID NO: 54, 55, 56, or 57. This method involves (a) amplifying by PCR the human gene, or portion thereof, using oligonucleotide primers that (i) are each greater than about 12 residues in length, and (ii) each have regions of complementarity to opposite DNA strands in a region of the nucleotide sequence of Figs. 13A or 13B, and (b) isolating the human gene, or portion thereof. This method can also include a step of testing the gene, or portion thereof, for the ability to functionally complement a *C. elegans daf-16* mutant.

In another aspect, the invention features a method of determining whether a human gene is involved in an impaired glucose tolerance condition (for example, a condition involving atherosclerosis) or obesity. This method involves (a) providing a nematode having a mutation in a *daf* or *age* gene, and (b) expressing in the nematode the human gene, which is operatively linked to a nematode gene promoter. Complementation of the *daf* or *age* mutation in the nematode is indicative of a human gene that is involved in an impaired glucose tolerance condition or obesity. In preferred embodiments, the nematode gene promoter is selected from the group consisting of *daf-1*, *daf-3*, *daf-4*, *daf-2*, *age-1*, and *akt* gene promoters. In other preferred embodiments, the *daf* mutation is selected from

the group consisting of *daf-2*, *daf-3*, *daf-1*, *daf-4*, *daf-7*, *daf-8*, *daf-11*, *daf-12*, *daf-14*, and *daf-16* mutations. In yet another preferred embodiment, the mutation can also be found in the *age-1* gene.

In further aspects, the invention features methods for diagnosing an impaired glucose tolerance condition (for example, Type II diabetes or a condition involving atherosclerosis), or a propensity for such a condition, in a patient. One such method includes analyzing the DNA of the patient to determine whether the DNA contains a mutation in a *daf* gene. Identification of such a mutation indicates that the patient has an impaired glucose tolerance condition or a propensity for such a condition. The analysis in this method can be carried out, for example, by nucleotide sequencing or RFLP analysis. The analysis can also include amplifying (for example, by PCR or reverse transcriptase PCR) the gene (for example, a human gene), or a fragment thereof, using primers, and analyzing the amplified gene, or a fragment thereof, for the presence of the mutation. In preferred embodiments, the *daf* gene analyzed in this method is, for example, a *daf-1*, *daf-2*, *daf-3*, *daf-4*, *daf-7*, *daf-8*, *daf-11*, *daf-12*, *daf-14*, or *daf-16* coding sequence, or the *daf* gene is FKHR or AFX.

Another method for diagnosing an impaired glucose tolerance condition, such as Type II diabetes, or a propensity for such a condition, in a patient, includes analyzing the DNA of the patient to determine whether the DNA contains a mutation in an *age* gene. Identification of such a mutation indicates that the patient has an impaired glucose tolerance condition or a propensity for such a condition. The analysis in this method can be carried out, for example, by nucleotide sequencing or RFLP analysis. The analysis can also include amplifying (for example, by PCR or reverse transcriptase PCR) the gene (for example, a human gene), or a fragment thereof, using primers and analyzing the amplified gene, or fragment thereof, for the presence of the mutation. In a preferred

embodiment, the *age* gene is an *age-1* coding sequence.

Yet another method for diagnosing an impaired glucose tolerance condition, such as Type II diabetes or a condition that involves atherosclerosis, or a propensity for such a condition, in a patient, includes analyzing the DNA of the patient to determine whether the DNA contains a mutation in an *akt* gene. Identification of such a mutation indicates that the patient has an impaired glucose tolerance condition (for example, Type II diabetes) or a propensity for such a condition (for example, a pre-diabetic condition). The analysis in this method can be carried out, for example, by nucleotide sequencing or RFLP analysis. The analysis can also include amplifying (for example, by PCR or reverse transcriptase PCR) the gene (for example, a human gene), or a fragment thereof, using primers and analyzing the amplified gene, or fragment thereof, for the presence of the mutation.

In another aspect, the invention features methods for ameliorating or delaying the onset of an impaired glucose tolerance condition (for example, Type II diabetes) in a patient. In one such method a therapeutically effective amount of a DAF polypeptide (for example, the human or nematode DAF-7 polypeptide) is

administered to the patient. In another method, which can be used, for example, in the case of a condition involving atherosclerosis, a therapeutically effective amount of a compound that is capable of inhibiting the activity of a DAF-16 or DAF-3 polypeptide is administered to the patient. In yet another method, a therapeutically effective amount of a compound that activates a DAF-1, DAF-4, DAF-8, DAF-11, or DAF-14 polypeptide is administered to the patient.

Another aspect of the invention provides methods for ameliorating or preventing obesity (for example, obesity associated with Type II diabetes) in a patient. One such method involves administering to the patient a therapeutically effective amount of a DAF polypeptide, such as a human or nematode DAF-7 polypeptide. Another such method involves administering to the patient a therapeutically effective amount of a compound that is capable of inhibiting the activity of a DAF-16 or DAF-3 polypeptide.

Yet another aspect of the invention features a transgenic, non-human animal, such as a mouse or a nematode, whose germ cells and somatic cells contain a transgene coding for a mutant DAF polypeptide, for example, a mutant DAF polypeptide that is derived from a human. In preferred embodiments, the mutant DAF polypeptide is a DAF-1, DAF-2, DAF-3, DAF-4, DAF-7, DAF-8, DAF-11, DAF-12, DAF-14, or DAF-16 polypeptide. In another preferred embodiment, the transgene includes a knockout mutation.

In a related aspect, the invention features a transgenic, non-human animal, such as a mouse or a nematode, whose germ cells and somatic cells contain a transgene coding for a mutant AGE polypeptide, for example, a mutant AGE polypeptide derived from a human. In a preferred embodiment, the mutant AGE polypeptide is an AGE-1 polypeptide. In another preferred embodiment, the transgene includes a knockout mutation.



mutation in an *akt* gene, and (b) introducing a transgene that (i) encodes a human AKT polypeptide, and (ii) is capable of expressing the human polypeptide, into an embryonal cell of the non-human animal.

Another aspect of the invention features a method of screening for a compound that increases the activity of a DAF polypeptide. This method includes (a) exposing a non-human transgenic animal whose germ cells and somatic cells contain a transgene coding for a mutant DAF polypeptide to a candidate compound, and (b) determining the activity of the DAF polypeptide in the transgenic animal. An increase in DAF polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of increasing DAF polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition or obesity.

In a related aspect, the invention features a method of screening for a compound that decreases the activity of a DAF polypeptide. This method includes (a) exposing a non-human transgenic animal whose germ cells and somatic cells contain a transgene coding for a mutant DAF polypeptide to a candidate compound, and (b) determining the activity of the DAF polypeptide in the transgenic animal. A decrease in DAF polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of decreasing DAF polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition, obesity, or atherosclerosis. In other preferred embodiments, the compound decreases the activity of DAF-3 or DAF-16.

In another aspect, the invention features a method of screening for a compound that increases the activity of an AGE polypeptide. This method includes (a) exposing a non-human transgenic animal whose germ cells and somatic cells contain a transgene coding for a mutant AGE polypeptide to a

candidate compound, and (b) determining the activity of the AGE polypeptide in the transgenic animal. An increase in AGE polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of increasing AGE polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition, obesity, or atherosclerosis.

In a related aspect, the invention features a method of screening for a compound that decreases the activity of a AGE polypeptide. This method includes (a) exposing a non-human, transgenic animal whose germ cells and somatic cells contain a transgene coding for a mutant AGE polypeptide to a candidate compound, and (b) determining the activity of the AGE polypeptide in the transgenic animal. A decrease in AGE polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of decreasing AGE polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition, obesity, or atherosclerosis. In another preferred embodiment, the AGE polypeptide is AGE-1.

In another aspect, the invention features a method of screening for a compound that increases the activity of an AKT polypeptide. This method includes (a) exposing a transgenic, non-human animal whose germ cells and somatic cells contain a transgene coding for a mutant AKT polypeptide to a candidate compound, and (b) determining the activity of the AKT polypeptide in the transgenic animal. An increase in AKT polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of increasing AKT polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition, obesity, or atherosclerosis.

In a related aspect, the invention features a method of screening for a compound that decreases the activity of a AKT polypeptide. This method includes (a) exposing a transgenic, non-human animal whose germ cells and somatic cells



contain a transgene coding for a mutant AKT polypeptide to a candidate compound, and (b) determining the activity of the AKT polypeptide in the transgenic animal. A decrease in AKT polypeptide activity, as compared to untreated controls, is indicative of a compound that is capable of decreasing AKT polypeptide activity. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition or obesity.

Also included in the invention is a method of screening for a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) exposing a transgenic, non-human animal whose germ cells and somatic cells contain a transgene coding for a mutant DAF, AGE, or AKT polypeptide to a candidate compound, and (b) monitoring the blood glucose level of the animal. A compound that promotes maintenance of a physiologically acceptable level of blood glucose in the animal, as compared to untreated controls, is indicative of a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition. In a preferred embodiment, the compound can be used to treat Type II diabetes.

Another method of screening for a compound that is capable of ameliorating or delaying obesity is also included in the invention. This method involves (a) exposing a transgenic, non-human animal whose germ cells and somatic cells contain a transgene coding for a mutant DAF, AGE, or AKT polypeptide to a candidate compound, and (b) monitoring the adipose tissue of the animal. A compound that promotes maintenance of a physiologically acceptable level of adipose tissue in the animal, as compared to untreated controls, is indicative of a compound that is capable of ameliorating or delaying obesity.

A related method of the invention can be used for screening for a compound that is capable of ameliorating or delaying atherosclerosis. This method involves (a) exposing a transgenic, non-human animal whose germ cells and somatic cells

contain a transgene coding for a mutant DAF, AGE, or AKT polypeptide to a candidate compound, and (b) monitoring the adipose tissue of the animal. A compound that promotes maintenance of a physiologically acceptable level of adipose tissue in the animal, as compared to untreated controls, is indicative of a compound that is capable of ameliorating or delaying atherosclerosis.

In another aspect, the invention includes a method for identifying a modulatory compound that is capable of decreasing the expression of a *daf* gene. This method involves (a) providing a cell expressing the *daf* gene, and (b) contacting the cell with a candidate compound. A decrease in *daf* expression following contact with the candidate compound identifies a modulatory compound. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition or obesity. In other preferred embodiments, the compound is capable of decreasing the expression of DAF-3 or DAF-16. This method can be carried out in an animal, such as a nematode.

In a related aspect, the invention includes a method for the identification of a modulatory compound that is capable of increasing the expression of a *daf* gene. This method involves (a) providing a cell expressing the *daf* gene, and (b) contacting the cell with a candidate compound. An increase in *daf* expression following contact with the candidate compound identifies a modulatory compound. In preferred embodiments, the compound can be used to treat an impaired glucose tolerance condition or obesity. In other preferred embodiments, the compound is capable of increasing expression of DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, or DAF-14. This method can be carried out in an animal, such as a nematode.

In another aspect, the invention includes a method for the identification of a modulatory compound that is capable of increasing the expression of an *age-1* gene. This method involves (a) providing a cell expressing the *age-1* gene, and (b)

contacting the cell with a candidate compound. An increase in *age-1* expression following contact with the candidate compound identifies a modulatory compound. In preferred embodiments, the compound is capable of treating an impaired glucose tolerance condition or obesity. This method can be carried out in an animal, such as a nematode.

In another aspect, the invention provides a method for identification of a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) providing a dauer larvae including a mutation in a *daf* gene, and (b) contacting the dauer larvae with a compound. Release from the dauer larval state is an indication that the compound is capable of ameliorating or delaying an impaired glucose tolerance condition. In a preferred embodiment, the dauer larvae carries a *daf-2* mutation. In another preferred embodiment, the dauer larvae is from *C. elegans*. In yet another embodiment, the impaired glucose tolerance condition involves obesity or atherosclerosis.

In a related aspect, the invention provides a method for identification of a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) providing a dauer larvae including a mutation in an *age-1* gene, and (b) contacting the dauer larvae with a compound. Release from the dauer larval state is an indication that the compound is capable of ameliorating or delaying an impaired glucose tolerance condition. In a preferred embodiment, the dauer larvae carries an *age-1* mutation. In another preferred embodiment, the dauer larvae is from *C. elegans*. In yet another preferred embodiment, the impaired glucose tolerance condition involves obesity or atherosclerosis.

In another related aspect, the invention provides a method for the identification of a compound that is capable of ameliorating or delaying an impaired glucose tolerance condition. This method involves (a) providing a dauer



whereby the nematode forms a dauer larva, and (c) contacting the dauer larva with a compound. A release from the dauer larval state is an indication that the compound is capable of ameliorating or delaying the glucose intolerance condition.

As used herein, by a “DAF” polypeptide is meant a polypeptide that functionally complements a *C. elegans daf* mutation and/or that has at least 60%, preferably 75%, and more preferably 90% amino acid sequence identity to a 100 amino acid region (and preferably a conserved domain) of a *C. elegans* DAF polypeptide. Complementation may be assayed in an organism (for example, in a nematode) or in a cell culture system. Complementation may be partial or complete, but must provide a detectable increase in function (as described herein). DAF polypeptides are encoded by “DAF” genes or nucleic acid sequences.

By an “AGE” polypeptide is meant a polypeptide that functionally complements a *C. elegans age* mutation and/or that has at least 60%, preferably 75%, and more preferably 90% amino acid sequence identity to a 100 amino acid region (and preferably a conserved domain) of a *C. elegans* AGE polypeptide. Complementation may be assayed in an organism (for example, in a nematode) or in a cell culture system. Complementation may be partial or complete, but must provide a detectable increase in a known AGE function. AGE polypeptides are encoded by “AGE” genes or nucleic acid sequences.

As used herein, by an “AKT” polypeptide is meant a polypeptide that functionally complements a *C. elegans akt* mutation and/or that possess at least 64% amino acid sequence identity to SEQ ID NO: 60, at least 71% amino acid sequence identity to SEQ ID NO: 61, at least 79% amino acid sequence identity to SEQ ID NO: 62, at least 63% amino acid sequence identity to SEQ ID NO: 63, at least 48% amino acid sequence identity to SEQ ID NO: 64, at least 70% amino acid sequence identity to SEQ ID NO: 65, at least 64% amino acid sequence

identity to SEQ ID NO: 66, at least 67% amino acid sequence identity to SEQ ID NO: 67, or a combination thereof. Complementation may be assayed in an organism (for example, in a nematode) or in a cell culture system. Complementation may be partial or complete, but must provide a detectable increase in a known AKT function. AKT polypeptides are encoded by “AKT” genes or nucleic acid sequences.

By a “DAF-2 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-2* mutation and/or that possesses at least 61% amino acid sequence identity to SEQ ID NO: 33, at least 31% amino acid sequence identity to SEQ ID NO: 34, at least 43% amino acid sequence identity to SEQ ID NO: 79, at least 35% amino acid sequence identity to SEQ ID NO: 80, at least 35% amino acid sequence identity to SEQ ID NO: 81, at least 48% amino acid sequence identity to SEQ ID NO: 82, at least 43% amino acid sequence identity to SEQ ID NO: 83, at least 40% amino acid sequence identity to SEQ ID NO: 84, or a combination thereof. Preferably, a DAF-2 polypeptide includes an aspartic acid, a proline, a proline, a serine, an alanine, an aspartic acid, a cysteine, or a proline at amino acid positions corresponding to *C. elegans* DAF-2 amino acids 1252, 1312, 1343, 347, 451, 458, 526, 279, and 348 respectively, or a combination thereof.

By a “DAF-3 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-3* mutation and/or that possesses at least 60% amino acid sequence identity to SEQ ID NO: 35, at least 38% amino acid sequence identity to SEQ ID NO: 36, at least 47% amino acid sequence identity to SEQ ID NO: 85, or a combination thereof. Preferably, a DAF-3 polypeptide includes a proline or a glycine at amino acid positions corresponding to *C. elegans daf-3* amino acids at positions 200 (proline) and/or 620 (glycine) in Fig. 12A, respectively, or a combination thereof. For example, the polypeptide may include a proline in the motif GRKGFPHV or a glycine in the motif RXXIXXG (where X

is any amino acid).

By a “DAF-16 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-16* mutation and/or that possesses at least 71% amino acid sequence identity to SEQ ID NO: 54, at least 35% amino acid sequence identity to SEQ ID NO: 55, at least 65% amino acid sequence identity to SEQ ID NO: 56, at least 53% amino acid sequence identity to SEQ ID NO: 57, or a combination thereof. In addition, a DAF-16 polypeptide preferably includes a serine residue in the conserved motif WKNSIRH (SEQ ID NO: 59).

By a “DAF-7 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-7* mutation and/or that possesses at least 29% amino acid sequence identity to SEQ ID NO: 26, at least 66% amino acid sequence identity to SEQ ID NO: 27, at least 45% amino acid sequence identity to SEQ ID NO: 28, at least 33% amino acid sequence identity to SEQ ID NO: 29, at least 56% amino acid sequence identity to SEQ ID NO: 30, at least 75% sequence identity to SEQ ID No: 51, or a combination thereof. Preferably, a DAF-7 polypeptide includes a proline or a glycine at amino acid positions corresponding to *C. elegans daf-7* amino acids 271 and 280, respectively, or a combination thereof.

By a “DAF-8 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-8* mutation and/or that possesses at least 46% amino acid sequence identity to SEQ ID NO: 23, at least 45% amino acid sequence identity to SEQ ID NO: 24, at least 36% amino acid sequence identity to SEQ ID NO: 25, or a combination thereof.

By an “AGE-1 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans age-1* mutation (previously known as a *daf-23* mutation) and/or that possesses at least 40% amino acid sequence identity to SEQ ID NO: 17, at least 45% amino acid sequence identity to SEQ ID NO: 18, at least 30% amino acid sequence identity to SEQ ID NO: 19, at least 24% amino acid

sequence identity to SEQ ID NO: 38, or a combination thereof. Preferably, an AGE-1 polypeptide includes an alanine at amino acid positions corresponding to *C. elegans age-1* amino acids 845.

By a “DAF-1 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-1* mutation and/or that possesses at least 45% amino acid sequence identity to SEQ ID NO: 13, at least 35% amino acid sequence identity to SEQ ID NO: 14, at least 65% amino acid sequence identity to SEQ ID NO: 15, at least 25% amino acid sequence identity to SEQ ID NO: 16, or a combination thereof. Preferably, a DAF-1 polypeptide includes a proline at the amino acid position corresponding to *C. elegans* DAF-1 amino acid 546.

By a “DAF-4 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-4* mutation and/or that possesses at least 45% amino acid sequence identity to SEQ ID NO: 20, at least 40% amino acid sequence identity to SEQ ID NO: 21, at least 44% amino acid sequence identity to SEQ ID NO: 22, or a combination thereof.

By a “DAF-11 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-11* mutation and/or that possesses at least 40% amino acid sequence identity to SEQ ID NO: 75, at least 43% amino acid sequence identity to SEQ ID NO: 76, at least 36% amino acid sequence identity to SEQ ID NO: 77, at least 65% amino acid sequence identity to SEQ ID NO: 78, or a combination thereof.

By a “DAF-12 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-12* mutation and/or that possesses at least 42% amino acid sequence identity to SEQ ID NO: 72, at least 58% amino acid sequence identity to SEQ ID NO: 73, at least 34% amino acid sequence identity to SEQ ID NO: 74, or a combination thereof.



By a “DAF-14 polypeptide” is meant a polypeptide that complements (as defined above) a *C. elegans daf-14* mutation and/or that possesses at least 48% amino acid sequence identity to SEQ ID NO: 68, at least 37% amino acid sequence identity to SEQ ID NO: 69, at least 48% amino acid sequence identity to SEQ ID NO: 70, at least 37% amino acid sequence identity to SEQ ID NO: 71, or a combination thereof.

By “insulin receptor activity” is meant any activity exhibited by an insulin receptor and measured by either (i) activation of insulin receptor substrate-1 (IRS-1) phosphorylation and recruitment of PI-3 kinase, (ii) activation of glucose transporter (Glut 4) fusion with a cellular membrane and concomitant glucose uptake, or (iii) activation of glycogen and/or fat synthesis and concomitant inhibition of gluconeogenesis or lipolysis or both.

By “insulin receptor related activity” is meant any activity not directly attributable to the insulin receptor but that is measured by an activation of IRS-1 phosphorylation and recruitment of PI3-kinase.

By “IGF-1 receptor activity” is meant any activity exhibited by an insulin-like growth factor-1 receptor and measured by (i) activation of IRS-1 phosphorylation and recruitment of PI-3 kinase, (ii) activation of cell division in NIH3T3 cells (e.g., as described in Gronborg et al., J. Biol. Chem. 268: 23435-23440, 1993), or (iii) activation of bone growth in, for example, the mouse model.

By “SMAD protein” is meant a protein that is capable of coupling to TGF- $\beta$  type ser/thr receptors. Smad proteins typically contain a smad conserved motif as described by Derynk et al. (*Cell* 87: 173, 1996). Exemplary smad proteins include, without limitation, DAF-3, MADR-2, MAD, DPC-4, and Sma-2.

By “AKT activity” is meant any activity exhibited by an AKT polypeptide and measured by phosphatidylinositol-regulated increases in serine phosphorylation of GSK-3 or activation of non-dauer growth in *C. elegans akt*

mutants.

By "impaired glucose tolerance condition" is meant any condition in which blood sugar levels are inappropriately elevated or lack normal metabolic regulation. Examples of such conditions include, without limitation, Type I diabetes, Type II diabetes, and gestational diabetes, and may be associated with obesity and atherosclerosis.

By "protein" or "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

By "substantially pure" is meant a preparation which is at least 60% by weight (dry weight) the compound of interest, e.g., any of the polypeptides of the invention such as the DAF-2, DAF-3, or DAF-16 polypeptides or DAF-2, DAF-3, or DAF-16-specific antibodies. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

By "isolated DNA" is meant DNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By a "substantially identical" polypeptide sequence is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the function of the polypeptide (assayed, e.g., as described herein).

Preferably, such a sequence is at least 75%, more preferably 85%, and most preferably 95% identical at the amino acid level to the sequence used for comparison.

Homology is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705 or BLAST software available from the National Library of Medicine). Examples of useful software include the programs, Pileup and PrettyBox. Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By a "substantially identical" nucleic acid is meant a nucleic acid sequence which encodes a polypeptide differing only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the function of the polypeptide (assayed, e.g., as described herein). Preferably, the encoded sequence is at least 75%, more preferably 85%, and most preferably 95% identical at the amino acid level to the

sequence of comparison. If nucleic acid sequences are compared a "substantially identical" nucleic acid sequence is one which is at least 85%, more preferably 90%, and most preferably 95% identical to the sequence of comparison. The length of nucleic acid sequence comparison will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides. Again, homology is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of any of the polypeptides disclosed herein including, but not limited to, DAF-2, DAF-3, and DAF-16 and any human homolog thereof).

By "purified antibody" is meant antibody which is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, antibody.

By "specifically binds" is meant an antibody which recognizes and binds a polypeptide of the invention (e.g., DAF-2, DAF-3, and DAF-16) but which does not substantially recognize and bind other molecules in a sample (e.g., a biological sample) which naturally includes a polypeptide of the invention. An antibody which "specifically binds" such a polypeptide is sufficient to detect protein product in such a biological sample using one or more of the standard immunological techniques available to those in the art (for example, Western blotting or immunoprecipitation).

By "immunological methods" is meant any assay involving antibody-based detection techniques including, without limitation, Western blotting, immunoprecipitation, and direct and competitive ELISA and RIA techniques.

By "means for detecting" is meant any one or a series of components that sufficiently indicate a detection event of interest. Such means involve at least one label that may be assayed or observed, including, without limitation, radioactive, fluorescent, and chemiluminescent labels.

By "hybridization techniques" is meant any detection assay involving specific interactions (based on complementarity) between nucleic acid strands, including DNA-DNA, RNA-RNA, and DNA-RNA interactions. Such hybridization techniques may, if desired, include a PCR amplification step.

By a "modulatory compound", as used herein, is meant any compound capable of either decreasing DAF-3 and DAF-16 expression (i.e., at the level of transcription, translation, or post-translation) or decreasing DAF-3 and DAF-16 protein levels or activity. Also included are compounds capable of either increasing DAF-1, DAF-2, DAF-4, DAF-8, DAF-7, DAF-11, DAF-14, AGE-1, and AKT expression (i.e., at the level of transcription, translation, or post-translation) or increasing DAF-1, DAF-2, DAF-4, DAF-8, DAF-7, DAF-11, DAF-14, AGE-1, and AKT protein levels or their corresponding activities.

By "complementation" is meant an improvement of a genetic defect or mutation. In one example, complementation of a genetic defect in a *daf*, *age*, or *akt* gene can be carried out by providing the wild-type *daf*, *age*, or *akt* genes, respectively. Complementation is generally accomplished by expressing the wild-type version of the protein in a host cell or animal bearing a mutant or inactive version of the gene.

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

### Detailed Description

The drawings will first be described.

#### Drawings

**Fig. 1** shows the genetic and physical map of *C. elegans daf-2*. The top panel shows the genetic map of *daf-2*. *daf-2* maps on the left arm of chromosome III 11.4 map units to the right of *dpy-1* and 1.6 map units to the left of *ben-1* (ACeDB). The middle panel shows the physical map of *daf-2*. *daf-2* maps between *mgP34* and *mgP44* in a region not covered by cosmid clones but covered by YAC Y53G8. Cosmids from the approximate *daf-2* genetic location detect RFLPs between *C. elegans* strains Bristol N2 and Bergerac RC301. *mgP31* on cosmid T21A6 is a HindIII RFLP: 5.3 kb in Bristol, 4.5 kb in RC301. *mgP33* on cosmid T02B2 is a HindIII RFLP: 9 kb in Bristol, 8 kb in RC301. *mgP34* on cosmid R10F2 is an EcoRI RFLP: 4.1 and 2.8 kb in Bristol, 3.6 kb in RC301. *mgP44* on cosmid R07G11 is a complex EcoRI RFLP: 2.9 kb, 2.4 kb, 1.9 kb and 1.7kb in Bristol; 3.6kb, 2.5kb and 1.6kb in RC301. *mgP35* on cosmid T10D5 is a StyI RFLP: 5.4 kb in Bristol, 5.8 kb in RC301. *mgP32* on cosmid C42B8 is a StyI RFLP: 2.8 kb in Bristol; 2.9kb in RC301. *mgP48* detected with *daf-2* probe (nt 1277-2126 and 3747-4650) is a HindIII RFLP: 4.3kb and 7kb in Bristol and 4.1kb and 6.2kb in RC301. Thirty-one out of thirty-three Dpy-non-Daf recombinants carry the RC301 allele of *mgP34* whereas all thirty-three recombinants in this interval carry the RC301 allele of *mgP44*, mapping *daf-2* 0.69 map units to the right of *mgP34* and to the left of *mgP44*. Fourteen out of twenty-four Ben-non-Daf recombinants carry the RC301 *mgP44* allele whereas all of these recombinants carry the RC301 allele of *mgP34*, mapping *daf-2* 0.66 map units to the left of *mgP44*.

Y53G8 YAC DNA was isolated from CHEF gels as described in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York,



TGATGCGAACGGCGATCGAT (SEQ ID NO: 7) and TGAGGGCCAACTAAAGAAGAC (SEQ ID NO: 8) were used. In the kinase domain primers CGCTACGGCAAAAAAGTGAA (SEQ ID NO: 9) and GACGATCCCGAGGTGAGTAT (SEQ ID NO: 10) were used. The presence of an SL1 spliced leader sequence indicates a full length *daf-2* cDNA. The predicted ORF is shown as a box; 5' and 3' UTRs are shown as thick bars. The predicted DAF-2 initiator methionine at base 486 is preceded by an in frame stop codon 63 bases upstream. The predicted DAF-2 stop codon is found at base 5658. No consensus polyadenylation signal was found in the cDNA nor in genomic shotgun sequence #00678, which extends 302 bp further downstream. The initial insulin receptor homolog shotgun sequences are shown as thin bars above the box.

Introns were detected by a combination of *in silico* genomic and cDNA sequence comparison, and by comparison of PCR products derived from cDNA and genomic DNA templates. The open triangles over a vertical bar indicate positions of the detected exon/intron boundaries. All the intron donor sites have GT consensus and the acceptor sites have AG consensus (Krause, 1995 *supra*). The triangles without a vertical bar indicate the approximate intron locations determined by comparison of PCR products using genomic DNA or cDNA as a template. Intron lengths were estimated by comparison of the PCR product size using cDNA or genomic DNA templates. Genomic regions corresponding to some of the introns could not be PCR amplified suggesting that these introns are long. The minimum *daf-2* gene size based on this analysis is 33 kb.

**Fig. 2A** shows the predicted *C. elegans* DAF-2 amino acid sequence. The predicted cysteine-rich region (amino acids 207-372) and tyrosine kinase domain (amino acids 1124-1398) are boxed. The signal peptide (amino acids 1-20), proteolysis site (amino acids 806-809), transmembrane domain (amino acids 1062-1085), and PTB binding motif in the juxtamembrane region (NPEY, amino



acids 1103-1106) are underlined. Three DAF-2 tyrosine residues, Y1293, Y1296 and Y1297, in the region corresponding to the insulin receptor kinase Y1158 to Y1163 activation loop are likely to be autophosphorylated, based on the predicted similarity between the DAF-2 and insulin receptor phosphorylation targets (Fig. 2B). Another likely target for DAF-2 autophosphorylation is the Y1106 NPEY motif located in the region corresponding to the insulin receptor juxtamembrane region NPEY motif (at Y972), that has been shown to mediate IRS-1 binding via its PTB domain to the insulin receptor (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). While DAF-2 bears one YXXM motif implicated in coupling to PI 3-kinase, mammalian IRS-1 and *Drosophila* insulin receptor (Fernandez et al., *EMBO J.* 14: 3373-3384, 1995) bear multiple YXXM motifs. Although no p85-like adaptor subunit has yet been detected in the *C. elegans* database, the AGE-1 homology to mammalian p110 suggests the existence of a homologous or analogous adaptor (Morris et al., *Nature* 382: 536-539, 1996). In the DAF-2 C-terminal domain, two other tyrosine residues may be autophosphorylated and bound to particular SH2-containing proteins: Y1678 binding to a PLC-g or SHP-2 homolog, and Y1686, perhaps binding to SEM-5 (Fig. 2A) (Songyang et al., *Cell* 72: 767-778, 1993). While mutations in, for example, ras and MAP kinase have not been identified in screens for dauer constitutive or dauer defective mutations, these general signaling pathway proteins may couple to DAF-2 as they couple to insulin signaling in vertebrates (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). The predicted phosphotyrosine residues in juxtamembrane region and the kinase domain activation loop are circled. In the extended C-terminal region, predicted phosphotyrosine residues are also circled and SH2-binding sites are underlined (see below).

**Fig. 2B** shows the cDNA encoding the *C. elegans* DAF-2.

**Fig. 2C** shows the amino acid comparison of *C. elegans* DAF-2 to the human insulin receptor and human IGF-I receptor (shown in parenthesis), and to the *Drosophila* insulin receptor homolog, with *daf-2* and human insulin receptor mutations highlighted. Six *daf-2* mutations map in the ligand-binding domain: *sa187* (C347S, TGT to AGT), *e1368* (S451L, TCA to TTA), *e1365* (A458T, GCT to ACT), *sa229* (D526N, GAT to AAT), and two mutations in *mg43* (C279Y, TGT to TAT and P348L, CCC to CTC). Three *daf-2* mutations substitute conserved amino acid residues in the insulin receptor kinase domain: *sa219* (D1252N, GAT to AAT), *e1391* (P1312L, CCC to CTC), and *e1370* (P1343S, CCA to TCA). Darkened residues indicate amino acid identity. Hatched residues indicate amino acid similarity. The percentages under the domains represents the percentage of identity observed between DAF-2 and each receptor. The corresponding BLAST probabilities of DAF-2 random match to each protein is:  $6.4 \times 10^{-157}$  (human insulin receptor),  $2.7 \times 10^{-156}$  (human IGF-I receptor),  $2.1 \times 10^{-153}$  (molluscan InR homolog),  $8.3 \times 10^{-153}$  (mosquito InR homolog),  $1.6 \times 10^{-138}$  (human insulin receptor-related receptor),  $1.7 \times 10^{-122}$  (*Drosophila* InR homolog),  $2.0 \times 10^{-108}$  (Hydra InR homolog). DAF-2 is more distant from the next most closely related kinase families:  $8.9 \times 10^{-58}$  (v-ros) and  $3.0 \times 10^{-51}$  (trkC neurotrophin receptor).

Conserved cysteine residues in the ligand-binding domain (top) are marked with dots. In the kinase domain, active site residues that mediate insulin receptor kinase specificity are marked with stars. All of these residues are homologous in DAF-2. The mutations found in human patients are indicated at the top of the row, and *daf-2* allele substitutions are indicated below with allele names. The sequence alignments were done with GCG programs, Pileup and Prettybox, and the identities were calculated with the GCG program, Gap.

**Fig. 3** is a photograph showing the metabolic control by *C. elegans* *daf-2* and *daf-7*. The top panel shows low levels of fat accumulation in a wild type L3

animal grown at 25°C that has been stained with Sudan black. Non-starved animals were fixed in 1% paraformaldehyde in PBS, frozen at -70°C, and freeze-thawed three times. Fixed animals were washed three times in PBS, and then incubated overnight in 1X Sudan black according to standard methods. The next panel shows higher levels of fat accumulation in *daf-2(e1370)* grown at the non-permissive temperature of 25°C. These animals accumulate fat in both intestinal and hypodermal cells. *daf-2(e1370)* animals grown at 15°C, the permissive temperature, accumulate low levels of fat, like wild type (data not shown). The next panel shows high fat levels in the intestine and hypodermis of *daf-7(e1372)* animals grown at 25°C. The bottom panel shows high levels of fat in *daf-2(e1370)* animals grown at the permissive temperature until the L4 stage and then shifted to the non-permissive temperature. This shows that *daf-2* regulates metabolism without entry into the dauer stage.

**Fig. 4** is a schematic diagram showing a model of insulin signaling in the *C. elegans* dauer formation pathway. In the absence of dauer pheromone, an insulin-like ligand activates DAF-2, and DAF-7 TGF- $\beta$ -like signal activates the DAF-1 and DAF-4 receptors. Activated DAF-2 autophosphorylates particular tyrosine residues and recruits signaling molecules, including the PI 3-kinase homolog (a heterodimer of an as yet unidentified p85 homolog and the PI 3-kinase catalytic subunit AGE-1). The AGE-1 PI 3-kinase produces PIP3 second messenger. This second messenger may regulate glucose transport (White and Kahn, 1994 *supra*), metabolic kinase cascades that include AKT and GSK-3 (Hemmings, *Science* 226:1344-1345, 1984; Jonas et al., *Nature*, 385:343-346, 1997), and transcription and translation of metabolic genes (White and Kahn, 1994, *supra*). DAF-16 acts downstream of DAF-2 and AGE-1 in this pathway and is negatively regulated by them (Vowels and Thomas, *Genetics*, 130:105-123, 1992; Gottlieb and Ruvkun, *Genetics*, 137:107-110, 1994). While both the

DAF-7/TGF- $\beta$  and DAF-2/insulin signaling pathways converge to control dauer formation, only the DAF-2 pathway controls reproductive phase longevity. This may be due to non-transcriptional outputs of DAF-2 suggested by precedents from insulin receptor signaling. DAF-7 signaling output is predicted to be only transcriptional as described herein.

**Fig. 5A** shows that *C. elegans daf-3* was genetically mapped to a region on the X chromosome between *aex-3* and *unc-1*. Cosmid and plasmid clones from the region were assayed for transformation rescue (Mello et al., *EMBO J* 10: 3959-3970, 1991). Plasmid pRF4 (*rol-6* transformation marker, 100 ng/ml), and cosmids (5-6 ng/ml) were injected into the gonad of *daf-7 (e1372)*; *daf-3 (e1376)* animals. Transgenic animals were scored for dauer formation at 25°C; a dauer (i.e., a return to the *daf-7* phenotype) indicates rescue of *daf-3*; clones that rescue *daf-3* are boxed. B0217 rescues the *daf-3* phenotype; eighteen of nineteen transgenic lines were rescued (~80% dauers). Examination of sequence provided by the *C. elegans* Sequencing Consortium revealed a Smad homologous gene on B0217. A 13 kb subclone of B0217 containing just the Smad also rescues *daf-3* (see Fig. 3). No rescue was seen upon injection of other cosmids from the region, B0504 (7 lines tested, <1% rescue) and C05H10 (10 lines tested, <1% rescue). *mgDf90* is a deletion that removes all of *daf-3*.

**Fig. 5B** shows the structure of the *C. elegans daf-3* coding region. The top is the exon/intron structure of *daf-3*; coding exons are filled boxes, non-coding regions are open boxes, and lines are introns. *daf-3* cDNAs were isolated according to standard methods. Four cDNAs were sequenced completely; their N-termini are indicated by vertical lines. These three cDNAs contain ~400 bp of 3' UTR, but no poly-A tail; a *C. elegans* consensus poly-adenylation sequence is found 12 bp from the 3' end of the cDNAs. The longest of this cDNA appears full-length, as it contains a methionine codon and the genomic sequence contains no

other methionine codon and no putative splice sites upstream before in-frame stop codons. To further characterize the 5' end of *daf-3*, PCR products from libraries or individual *daf-3* cDNAs were sequenced. From DNA isolated from a cDNA library, we amplified a product with a primer to SL1 and to a region in conserved domain I (shown as primer 1). For the individual cDNAs, we amplified with a primer to the cDNA vector and primer 1. These PCR products were sequenced from primer 2 to the 5' end, and we found that there is alternative splicing at the 5' end of *daf-3*, upstream of the conserved domains. The two alternate splice forms are indicated, and the ends of individual cDNAs are indicated by vertical lines. Note that the second has the trans-spliced leader SL1 that is found at the 5' end of many *C. elegans* cDNAs; thus, this cDNA shows a *bona fide* 5' end of *daf-3*.

**Fig. 5C** shows the protein sequence alignment of *C. elegans daf-3* and the closest homolog found to date, human DPC4, in the Smad conserved domains I and II. Dots indicate gaps introduced to maximize alignment. DAF-3 is 55% identical to DPC4 in domain I and 30% identical in domain II. *daf-3(mg125)* and *daf-3(mg132)* mutations are indicated by boldface and underline. The Smad mutational hotspot is underlined. In addition to *mg125* and *mg132*, seven other *daf-3* alleles were sequenced in the hotspot; none of them contains a mutation. Alleles sequenced were *mg91*, *mg93*, *mg105*, *mg121*, *mg126*, *mg133* (isolated by A. Kowek and G. Patterson, unpublished) and sa205.

**Figs. 6A-6G** is a panel of photographs showing *C. elegans* DAF-3 and DAF-4 expression. These photographs show GFP fluorescence, paired with DAPI fluorescence or Nomarski optics photographs, as marked. All DAF-3 photographs show animals with the second plasmid from **Fig. 6A** illustrates DAF-3/GFP head expression in an L1 animal. **Fig. 6B** illustrates DAF-3/GFP expression in the ventral nerve cord of an adult animal. L1 animals demonstrated similar expression patterns. **Fig. 6C** illustrates DAF-3/GFP expression in the intestine of an L1

animal. **Fig. 6D** illustrates DAF-3/GFP expression in the distal tip cell of an L4 animal. **Fig. 6E** illustrates DAF-3/GFP expression in an embryo with approximately 200 nuclei. **Fig. 6F** illustrates DAF-4/GFP expression in the head of an L1 animal. **Fig. 6G** illustrates DAF-4/GFP expression in the dorsal nerve cord and ventral nerve cord of an L4 animal.

**Fig. 7** is a table that shows the rescuing ability and suppression of *C. elegans daf-7* by *daf-3* plasmids. The solid boxes represent the Smad conserved domains I and II of *daf-3*; the stippled boxes represent green fluorescent protein (GFP). For all experiments shown, *daf-3* plasmids were injected at a concentration of 10 ng/ml, and the pRF4 injection marker was injected at a concentration of 90 ng/ml. To score dauer formation, transgenic adult animals were allowed to lay eggs on plates for several hours at room temperature and were then removed. The plates were scored after two days at 25°C. The rescue experiment shows the rescue of *daf-7(m62); daf-3(e1376)* by each of the fusion proteins. Failure to rescue results in rolling nondauers, while rescue of *daf-3* results in rolling dauers (the *daf-7* phenotype). The control is an array with the pRF4 transformation marker and a non-rescuing cosmid. For each construct, four or more lines were measured in two separate experiments. To measure suppression of *daf-7*, transgenic arrays were crossed into *daf-7* (for plasmids 1 and 3), or produced by injecting directly into *daf-7* (for plasmid 2). Transgenic (rolling) animals were scored for suppression of *daf-7* (= nondauers) or failure to suppress *daf-7* (= dauers). The controls are two array strains with the pRF4 marker and an unrelated GFP expressing transgene.

**Fig. 8A** is a photographs showing that DAF-3/GFP is associated with metaphase chromosomes. Fixed L1 animals were immunostained with anti-GFP antibody and anti- $\alpha$ -tubulin antibody. DNA was visualized using DAPI staining.

**Fig. 8B** is a photograph showing that a truncated *C. elegans daf-3*/GFP protein is predominantly nuclear. Wild-type animals were injected with the truncated construct shown in **Fig. 7** at a concentration of 10 ng/ml. The pRF4 transformation marker was injected at 100 ng/ml. The photograph shows a late L1 or early L2 animal, and *daf-3* is predominantly nuclear. The clear spot in the center of some of the nuclei is the nucleolus, which has no *daf-3*/GFP. All cells in these animals have predominantly nuclear *daf-3*/GFP, including the ventral cord neurons, intestinal cells, and distal tip cell (all shown), as well as head and tail neurons and hypodermal cells.

**Figs. 9A and 9B** show models for the role of the *C. elegans* *daf-3*/DAF-8/DAF-14 Smad proteins in dauer formation. **Fig. 9A** shows dauer reproductive growth induction. **Fig. 9B** shows reproductive dauer growth induction.

**Fig. 10** is a schematic illustration showing the genetic pathway that regulates *C. elegans* dauer formation.

**Figs. 11A-11C** show the cDNA sequences of the differentially spliced *C. elegans daf-3* transcripts (SEQ ID NOS: 39, 52, and 53).

**Figs. 12A-12C** show the amino acid sequences of the *C. elegans* DAF-3 polypeptide isoforms (SEQ ID NOS: 40-42).

**Figs. 13A and 13B** show the cDNA sequence of the differentially spliced *C. elegans daf-16* transcripts (SEQ ID NOS: 43 and 44).

**Figs. 14A and 14B** show the amino acid sequences of the *C. elegans* DAF-16 polypeptide isoforms (SEQ ID NOS: 45 and 46).

**Fig. 15** shows the cDNA sequence of the *C. elegans age-1* gene (SEQ ID NO: 47).

**Fig. 16** shows the amino acid sequence of the *C. elegans* AGE-1 polypeptide (SEQ ID NO: 48).

**Fig. 17** is a schematic diagram illustrating that convergent TGF- $\beta$  and insulin signaling activates glucose-based metabolic genes.

**Fig. 18** is a schematic diagram illustrating a switch to fat-based metabolism in the absence of DAF-7 and DAF-2 signals (in pheromone).

**Fig. 19** is a schematic diagram illustrating inhibition of the DAF-16 pathway by drugs to ameliorate lack of insulin signaling.

**Fig. 20** is a schematic diagram illustrating inhibition of DAF-3 by drugs to ameliorate a lack of DAF-7 signaling (for example in obesity-induced diabetes).

**Fig. 21A** is an illustration showing that human FKHR and AFX are the closest relatives to DAF-16. Note that the differentially spliced DAF-16 forkhead domain is less homologous.

**Fig. 21B** is an illustration showing a forkhead family tree, illustrating that DAF-16 is much more closely related to FKHR and AFX than any other forkhead protein.

**Fig. 22** is a photograph showing that *daf-16* is expressed in target tissues, like *daf-3*. This supports the model that DAF-3 and DAF-16 are capable of interacting.

**Fig. 23** is an illustration showing a model for treatment of obesity-induced diabetes with DAF-7 protein.

**Fig. 24** is an illustration showing the genetic mapping of *sup(mg144)* to the AKT genetic region.

**Fig. 25** is an illustration showing the comparison of *C. elegans* AKT with mammalian AKT.

**Fig. 26A** is a photograph showing the expression of AKT:GFP in *daf-2* dauers.

**Fig. 26B** is a photograph showing the expression of AKT:GFP in an N2 adult worm.



Fig. 27 is a schematic illustration showing the molecular map of *daf-16*.

**The DAF-2 Insulin Receptor Family Member Regulates Longevity and Diapause in *C. elegans***

Arrest at the *C. elegans* dauer stage is normally triggered by a dauer-inducing pheromone detected by sensory neurons which signal via a complex pathway to target tissues that are remodeled and metabolically shifted such as the germ line, intestine, and ectoderm (Riddle, In: *Caenorhabditis elegans* II, D. Riddle, T. Blumenthal, B. Meyer, J. Priess, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768. Kenyon, op cit., pp. 791-813.). Genetic epistasis analysis of *daf* mutants that arrest at the dauer stage or enter the reproductive life cycle independent of pheromone regulation has revealed parallel genetic pathways that regulate distinct aspects of the dauer metamorphosis (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). The pathway that includes *daf-2* is unique in that it controls both reproductive development and normal senescence: *daf-2* mutant animals arrest development at the dauer larval stage and have dramatically increased longevity (Table I) (Riddle, In: *Caenorhabditis elegans* II, D. Riddle, T. Blumenthal, B. Meyer, J. Priess, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768; Kenyon, op cit. pp 791-813; Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994; Larsen et al., *Genetics* 139: 1567-1583, 1995; Kenyon et al., *Nature* 366: 461-464, 1993; Dorman et al., *Genetics* 141: 1399-1406, 1995).

Table I shows the percentage of dauer formation of *daf-2* alleles and the associated mutations. Eggs from animals grown at 15°C (day 0) were incubated at 15, 20, or 25°C. Numbers in parenthesis are animals counted. Numbers of wild-

type animals and dauers were counted on day 3 (20°C and 25°C) or day 5 (15°C). Most of the dauers marked with stars recovered by day 4 (*sa229* at 25°C) or by day 8 (*sa229*) and *sa219* at 15°C, *el368* and *sg219* at 20°C, and *el365* and *el368* at 25°C). *mg43* was studied as follows: *dpy-1(el)daf-2(mg43)*; *SDP3* animals were grown at 20°C until the young adult stage. Eggs from five adults were laid at 15°C or 20°C and grown at the same temperatures. Numbers of Dpy-Daf animal and Dpy-non-Daf animals were counted on day 3 (20°C) or day 5 (15°C). *Sg187* and *sg229* were also studied by Malone and Thomas (*Genetics* 136:879-886, 1994).

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Table I. Percentage of dauer formation of *daf-2* alleles

Region	Allele	mutation	% dauer formation		
			15°C	20°C	25°C
cys-rich	mg43	C279Y&P34 8L	100.0 (215)	100.0 (245)	n.d.
	sa187	C347S	0.4 (461)	98.7 (224)	100 (910)
	e1368	S451L	0.0 (328)	4.5* (418)	99.7* (698)
ligand-binding	e1365	A458T	0.0 (450)	0.0 (461)	99.4* (814)
kinase	sa229	D526N	3.4* (234)	n.d.	22.1* (420)
	sa219	D1252N	10.0* (460)	99.7* (396)	100 (514)
	e1391	P1312L	3.3 (332)	100 (323)	100 (322)
	e1370	P1343S	0.0 (520)	0.0 (188)	100 (635)

Genetic mapping using both visible genetic markers and restriction fragment length polymorphism (RFLP) markers places *daf-2* between *mgP34* and *mgP44* (Fig. 1). While cosmid coverage of this physical genetic region is not complete, YAC Y53G8 carries the genomic region that includes *mgP34* and *mgP44*, which flank *daf-2* (Fig. 1). As a step in the *C. elegans* genome sequencing effort, random M13 subclones derived from Y53G8 were sequenced by the Genome Sequencing Center.

**Sequence Identities Show that DAF-2 is Likely to Bind to an Insulin-like Ligand and to Phosphorylate Tyrosine Residues**

The amino acid sequences and nucleotide sequences encoding DAF-2 are shown in Figs. 2A and 2B, respectively. Using BLASTX to compare 570 translated Y53G8 M13 subclone sequences against the Genbank protein database, we found that four sequences are homologous to the mammalian insulin receptor family. An insulin receptor was a good *daf-2* candidate gene because insulin regulates vertebrate growth and metabolism (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994), and because a phosphatidylinositol (PI) 3-kinase has been shown to act in both the insulin receptor and *daf-2* pathways (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994; Morris et al., *Nature* 382: 536-539, 1996). The detection of multiple *daf-2* mutations in the gene (see below), and the coincidence of the genetic location of this insulin receptor homolog with *daf-2* (Fig. 2C) establish that this insulin receptor homolog corresponds to *daf-2*.

The *daf-2* transcription unit and gene structure were determined using PCR primers derived from *daf-2* genomic subclone sequences to amplify *daf-2* genomic and cDNA regions. A probable full length *daf-2* cDNA bears a 5172 base open reading frame, a 485 base 5' UTR and a 159 base 3' UTR (Figs. 1, 2A). The predicted DAF-2 protein shows long regions of sequence identity to the insulin receptor family. Over the entire protein, DAF-2 is 35% identical to the human insulin receptor (Ebina et al., *Cell* 40: 747-58, 1985; Ullrich, et al., *Nature* 313: 756-61, 1985), 34% identical to the human IGF-I receptor (Ullrich, et al., *EMBO J.* 5: 2503-12, 1986), and 33% identical to the human insulin receptor-related receptor (Shier and Watt, *J. Biol. Chem.* 264: 14605-8, 1989). DAF-2 is the only member of the insulin receptor family in the 90 Mb *C. elegans* genome sequence (about 90% complete) or in the 10 Mb *C. elegans* EST sequence database. Because it is equally distant from insulin, IGF-I, and insulin receptor-related

receptors, DAF-2 is probably the homolog of the ancestor of these duplicated and diverged receptors, and thus may subserve any or all of the functions of these mammalian receptors (see below). Like these receptors, DAF-2 has a putative signal peptide, a cysteine-rich region in the putative ligand binding domain, a putative proteolysis site, a transmembrane domain, and a tyrosine kinase domain. In addition, DAF-2 has a C-terminal region that may serve a function similar to the mammalian insulin receptor substrate-1 (IRS-1) (Figure 2; White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994).

In the approximately 500 amino acid ligand-binding domain of the insulin receptor, DAF-2 is 36% identical to insulin receptor and 35% identical to the IGF-I receptor. Twenty-one of twenty-three phylogenetically conserved cysteine residues in this domain are conserved in DAF-2 (Fig. 2C). The DAF-2 cys-rich region is 34% identical to human insulin receptor and 28% identical to the IGF-I receptor. Six *daf-2* mutations map in this domain (Fig. 2C, Table I). The *mg43* and *sal87* mutations substitute conserved residues in the cys-rich region (Fig. 2C). *daf-2(mg43)* carries two mutations which substitute conserved residues, which may explain the strength of this allele (non-conditional, Table I). Other substitutions at non-conserved residues cause less severe phenotypes (Table I). Insulin resistant and diabetic patients with mutations in the ligand binding domain of the human insulin receptor gene have been identified (Taylor, *Diabetes* 41: 1473-1490, 1992) (see below). These mutations impair receptor transport to cell surface, or insulin binding affinity, or both. The DAF-2 mutations in this domain might similarly decrease receptor signaling to cause dauer arrest.

Insulin receptors are  $\alpha 2, \beta 2$  tetramers proteolytically processed from a single precursor protein (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). DAF-2 bears a probable protease recognition site at a position analogous to the insulin receptor processing site (RVRR 806-809) (Yoshimasa et al., *J. Biol. Chem.* 265:

17230-17237, 1990).

The 275 amino acid DAF-2 tyrosine kinase domain is 70% similar and 50% identical to the human insulin receptor kinase domain. Upon insulin binding, the intracellular tyrosine kinase domain of the insulin receptor phosphorylates particular tyrosine residues flanked by signature amino acid residues (upstream acidic and downstream hydrophobic amino acids (Songyang and Cantley, *Trends Biochem. Sci.* 20: 470-475, 1995)) in the intracellular domain as well as on IRS-1 (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). Multiple DAF-2 tyrosine residues in these sequence contexts are likely autophosphorylation targets, including three tyrosines in a region similar to the insulin receptor activation loop and one in the juxtamembrane region as described above (Fig. 2C). Based on the crystal structure of the insulin receptor kinase domain bound to its activation loop, eight kinase domain residues mediate target site specificity (Hubbard et al., *Nature* 372: 746-754, 1994). In DAF-2 (but not in more distantly related receptor kinases), these residues are invariant (5/8) or replaced with similar amino acids (3/8: K to R, E to D) (Fig. 2C), suggesting that DAF-2 phosphorylates the same target tyrosine motifs as the insulin receptor kinase.

Three *daf-2* missense mutations substitute conserved amino acid residues in the kinase domain (Fig. 2C, Table I). All three mutations cause moderate to strong dauer constitutive phenotype, but none are as strong as the non-conditional alleles, for example, *mg43* (Table I). Human insulin receptor mutations in the kinase domain exhibit decreased kinase activity and cause severe insulin resistance and associated defects (Fig. 2C; Taylor, *Diabetes* 41: 1473-1490, 1992). Remarkably, a human diabetic insulin resistant patient bears the same amino acid substitution (P1178L) as *daf-2(e1391)* (Kim et al., *Diabetologia* 35: 261-266, 1992). This patient was reported to be heterozygous for this substitution. *daf-2(e1391)* is not dominant whereas it is a highly penetrance recessive mutation (Table I).

To test for dominance of *daf-2(e1391)*, using a genetically marked balancer chromosome, 105 dauers segregated from 485 *daf-2/+* parents as expected for a recessive mutations. The genotype of 76/77 of these animals was homozygous *daf-2(e1391)* whereas 1/77 of the dauers was *daf-2(e1391)/+*, indicating a less than 1% dominance. It is possible that in contrast to *C. elegans*, the P1178L mutation in humans is dominant, or that the patient carries a second insulin receptor mutation in *trans*, or carries mutations in other genes (for example, other complex type II diabetes loci) that enhance the dominance of P1178L (Bruning et al., *Cell* 88: 561-572, 1997).

### **AGE-1 PI 3-kinase is a Major DAF-2 Signaling Output**

Like the *Drosophila* insulin receptor homolog, DAF-2 has a long C-terminal extension that may function analogously to mammalian IRS-1 (Fernandez et al., *EMBO J.* 14: 3373-3384, 1995). In mammals, IRS-1 tyrosine residues are phosphorylated by the insulin receptor kinase, and these phosphotyrosines mediate binding to a variety of signaling proteins bearing SH2 domains (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994; Songyang et al., *Cell* 72: 767-778, 1993.). Many, but not all, of the DAF-2 C-terminal extension tyrosines bear flanking sequence motifs suggestive that they are autophosphorylated (Fig. 2A; Songyang and Cantley, *Trends Biochem. Sci.* 20: 470-475, 1995). Based on precedents from IRS-1 interactions with mammalian PI 3-kinases (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994), a YXXM motif at DAF-2 Y1504 is likely to mediate interaction with the AGE-1 PI 3-kinase, which acts in the same genetic pathway as *daf-2* (Fig. 4) (Morris et al., *Nature* 382: 536-539, 1996).

Three DAF-2 tyrosine residues, Y1293, Y1296 and Y1297, in the region corresponding to the insulin receptor kinase Y1158 to Y1163 activation loop are likely to be autophosphorylated, based on the predicted similarity between the

DAF-2 and insulin receptor phosphorylation targets (Fig. 2C). Another likely target for DAF-2 autophosphorylation is the Y1106 NPEY motif located in the region corresponding to the insulin receptor juxtamembrane region NPEY motif (at Y972), that has been shown to mediate IRS-1 binding via its PTB domain to the insulin receptor (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). While DAF-2 bears one YXXM motif implicated in coupling to PI 3-kinase, mammalian IRS-1 and *Drosophila* insulin receptor (Fernandez et al., *EMBO J.* 14: 3373-3384, 1995) bear multiple YXXM motifs. Although no p85-like adaptor subunit has yet been detected in the *C. elegans* database, the AGE-1 homology to mammalian p110 suggests the existence of a homologous or analogous adaptor (Morris et al., *Nature* 382: 536-539, 1996). In the DAF-2 C-terminal domain, two other tyrosine residues may be autophosphorylated and bound to particular SH2-containing proteins: Y1678 binding to a PLC- $\gamma$  or SHP-2 homolog, and Y1686, perhaps binding to SEM-5 (Fig. 2A) (Songyang et al., *Cell* 72: 767-778, 1993). While mutations in, for example, ras and MAP kinase have not been identified in screens for dauer constitutive or dauer defective mutations, these general signaling pathway proteins may couple to DAF-2 as they couple to insulin signaling in vertebrates (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994).

The insulin receptor also couples to other signaling pathways (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994); analogous DAF-2 phosphotyrosine residues may mediate these interactions (as described above). Thus, we suggest that tyrosines in the DAF-2 cytoplasmic domain are autophosphorylated upon ligand binding, and recruit the AGE-1 PI-3 kinase homolog (as well as other molecules) to signal reproductive development and normal senescence.



### *Metabolic Control by daf-2 in Control of Diapause and Aging*

Insulin and its receptor families play key roles in vertebrate (and by our evidence in invertebrates) metabolic and growth control (Kahn and Weir, eds., *Joslin's Diabetes Mellitus*, Lea & Febiger, 1994). Upon insulin release--by increasing blood glucose and autonomic inputs--insulin receptor engagement directs a shift in the activities of key metabolic enzymes, as well as changes in the transcription and translation of metabolic regulators in fat, liver, and muscle cells, all of which lead to assimilation of glucose into glycogen and fat (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). IGF-I is released from the liver in response to pituitary growth hormone, and mediates many of the growth and development responses to that endocrine signal (Mathews et al., *Proc Natl Acad Sci. U.S.A.* 83: 9343-7, 1986). Interestingly, lifespan is dramatically increased in dwarf mice with defects in growth hormone signaling, and presumably decreased IGF-I signaling as well (Brown-Borg et al., *Nature* 384: 33, 1996). No function for the insulin receptor-related receptor has yet been established, though it is expressed in conjunction with NGF receptor (Reinhardt et al., *J. Neurosci.* 14: 4674-4683, 1994).

Diapause arrest in general and dauer arrest in particular are associated with major metabolic changes (Tauber et al., *Seasonal Adaptation of Insects*, Oxford University Press, New York, NY, 1986), consistent with a model that *daf-2* acts in a metabolic regulatory pathway related to insulin signaling. In wild-type animals, DAF-2 signaling allows non-dauer reproductive growth, which is associated with utilization of food for growth in cell number and size, and small stores of fat (Fig. 3). In *daf-2* mutant animals, metabolism is shifted to the production of fat (Fig. 3) and glycogen (data not shown) in intestinal and hypodermal cells. Even when a temperature-sensitive *daf-2* mutant allele is shifted to the non-permissive temperature at the L4 or adult stage (after the critical period for *daf-2* control of

dauer formation), metabolism is shifted towards storage of fat (Fig. 3). Thus *daf-2* also regulates metabolism during reproductive development. Similar metabolic shifts are seen in wild-type pheromone-induced dauers (data not shown), *age-1* mutants (data not shown), and *daf-7* mutants (Fig. 3). In support of this metabolic shift, in dauer larvae, enzymes that regulate glycolysis are down-regulated while those that regulate glycogen and fat synthesis are up-regulated, and there is ultrastructural evidence for increased lipid and glycogen (O'Riordan and Burnell, *Comp. Biochem. & Physiol.* 92B: 233-238, 1989; O'Riordan and Burnell, *Comp. Biochem. & Physiol.* 95B: 125-130, 1990; Popham and Webster, *Can. J. Zool.* 57: 794-800, 1978; Wadsworth and Riddle, *Develop. Biol.* 132: 167-173, 1989). The dauer metabolic shift is associated with arrest of germ line proliferation, and arrest of somatic cell division and enlargement (Riddle, In: *Caenorhabditis elegans* II, D. Riddle, T. Blumenthal, B. Meyer, J. Priess, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768; Kenyon, op cit., pp. 791-813).

There is precedent for insulin-like signaling in invertebrate metabolic and growth control: insulin-like growth factors have been detected in metabolism-regulating ganglia in molluscs (Roovers et al., *Gene* 162: 181-188, 1995) and regulate molting in locust (Hetru et al., *Eur. J. Biochem* 201: 495-499, 1991) and silkworm (Kawakami et al., *Science* 247: 1333-1335, 1990). Consistent with the *daf-2* regulation of diapause, injection of insulin into diapausing *Pieris brassicae* (an insect) pupae induces recovery (Arpagaus, *Roux's Arch. Dev. Biol.* 196: 527-530, 1987).

Without being bound to a particular theory, we hypothesize that an insulin-like signal is up-regulated during reproductive development and stimulates DAF-2 receptor autophosphorylation and recruitment of the AGE-1 PI 3-kinase to produce the second messenger PIP3. AGE-1 is likely to be a major signaling output of DAF-2 because of the similarity of the *age-1* and *daf-2* mutant

phenotypes and because of their similar placement in the epistasis pathway (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). Precedents from insulin receptor signaling suggest the following candidate targets for DAF-2/AGE-1/PIP3 regulation of metabolism: (1) membrane fusion of vesicles bearing glucose transporters (Kahn and Weir, eds., *Joslin's Diabetes Mellitus*, Lea & Febiger, 1994) (or more probably trehalose transporters (Tauber et al., *Seasonal Adaptation of Insects*, Oxford University Press, New York, NY, 1986)) to facilitate flux of this molecule for growth and reproductive metabolism; (2) PIP3 activates an AKT/GSK-3 kinase cascade (Hemmings, *Science* 275: 628-630, 1997) which may regulate the activities of glycogen and fat synthetic and lytic enzymes; (3) transcription and translation of metabolic genes such as PEPCK, GDH, fat synthetases, and lipases (White and Kahn, *J. Biol. Chem.* 269:1-4, 1994). Genetic epistasis analysis suggests that DAF-2/AGE-1 signaling negatively regulates *daf-16* gene activity (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). DAF-16 could act at any point downstream of AGE-1 in this signaling pathway. Evidence is presented herein that DAF-16 represents the major transcriptional output to DAF-2/AGE-1 PIP3 signaling.

In addition to these metabolic changes, the DAF-2 signaling cascade also controls the reproductive maturation of the germ line as well as morphogenetic aspects of the pharynx and hypodermis (Riddle, In: *Caenorhabditis elegans* II, D. Riddle, T. Blumenthal, B. Meyer, J. Priess, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768; Kenyon, op cit., pp. 791-813). The DAF-2 receptor may act, for example, in the hypodermal and intestinal target tissues where we note a change in metabolism triggered by the dauer regulatory cascade (Fig. 3). It is also possible that DAF-2 regulates the metabolism and remodeling of tissues indirectly, for example, by controlling the production of

other hormones (Nagasawa et al., *Science* 226: 1344-1345, 1984; Jonas, et al., *Nature* 385: 343-346, 1997). Expression and genetic mosaic analysis of *daf-2* is essential to distinguish these models.

Even though DAF-2 and the mammalian insulin receptor both regulate metabolism, the metabolic defects associated with mutations in these receptors appear to be different. Complete loss of mammalian insulin receptor activity causes growth arrest at birth (Leprechaunism in humans), and a metabolic shift to runaway lipolysis and ketoacidosis (Kahn and Weir, eds., *Joslin's Diabetes Mellitus*, Lea & Febiger, 1994), rather than the fat accumulation we observe in *daf-2* mutants (Fig. 3). This distinction between insulin receptor and *daf-2* mutants may reflect distinct metabolic responses to this signaling, or a difference between complete loss and declines in insulin signaling. In humans, ketoacidosis is only induced during severe starvation or pathological states when insulin levels are very low (Kahn and Weir, eds., *Joslin's Diabetes Mellitus*, Lea & Febiger, 1994). Since none of the *daf-2* mutations described herein are clear null mutations, it is possible that *daf-2* dauer-constitutive alleles are more analogous to non-null human insulin receptor mutations. Most *daf-2* alleles are temperature sensitive, including alleles isolated in genetic screens that would allow the recovery of non-temperature sensitive mutations (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). Substitutions of DAF-2 amino acid residues conserved across phylogeny cause more penetrant dauer arrest at all temperatures than substitutions of non-conserved residues. *daf-2* mutants that arrest development at the dauer stage independent of growth temperature are likely to have the least gene activity (for example *mg43*). Several *daf-2* alleles also cause 5% to 10% embryonic lethality (unpublished results), suggesting that *daf-2* functions during embryonic development. None of the *daf-2* mutations detected so far are nonsense, frameshift, or deletion alleles. It

is possible that the *daf-2* null phenotype is stronger than non-conditional dauer arrest, for example embryonic lethality. However, dauer constitutive *daf-2* mutant alleles are isolated from EMS mutagenesis at a very high rate (about 1/300 chromosomes), suggesting that the existing alleles are not rare viable alleles. In fact, the 14 year old patient with the same insulin receptor mutation as *daf-2(e1391)* was morbidly obese (Kim et al., *Diabetologia* 35: 261-266, 1992), suggesting that metabolic effects of decreased insulin signaling may be similar to *daf-2* mutants.

It may be significant to human diabetes that animals carrying mutations in *daf-16* can grow reproductively even if they also carry *daf-2* and *age-1* mutations that disable insulin-like metabolic control signals (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). These data suggest that it is unregulated *daf-16* gene activity that causes these metabolic shifts. The analogous metabolic defects associated with both type I and type II diabetes may be caused by similar unregulated activity of the human DAF-16 homolog. Below we disclose the molecular identity of *daf-16*. Inhibition of its activity is expected to ameliorate the metabolic dysregulation associated with insulin signaling defects.

### **DAF-16 Encodes a Forkhead Transcription Factor Homolog**

Using a combination of genetic mapping and detection of multiple *daf-16* mutations in a 5 kb region, we have determined the nucleic acid sequence of *daf-16*. *daf-16* was mapped 1 map unit to the left of *lin-11* and 3.3 map units right of *unc-75* on Chromosome I. This region of the genome contained a gap that was not covered by cosmids nor YACs. We used a cosmid library (Genome Sciences, Inc.) to walk into the gap. Sequence analysis of the ends of four fosmids (H27K20, H01H03, H12I08, and H35K06) revealed that the previously unmapped

contig 133 lies in the *lin-11 unc-75* gap. Cosmids from the approximate *daf-16* genetic location were used to detect RFLPs between *C. elegans* strains Bristol N2 and Bergerac RC301: *mgP45* on cosmid C39H11, *mgP46* on cosmid F28D9, *mgP49* on cosmid C35E7, *mgP50* is on cosmid C43H8. Zero out of 30 *daf* non-Unc recombinants carry the RC301 alleles of *mgP45* and *mgP50*. Two out of 30 *Daf* non-Unc recombinants carry the RC301 allele of *mgP49*. 10 out of 30 *Daf* non-Unc recombinants carry the RC301 allele of *mgP46*. 1 out of 4 non-Lin *Daf* recombinants carry the N2 allele of *mgP45*. 4 out of 4 non-Lin *Daf* recombinants carry the N2 allele of *mgP49*. These data indicate that *daf-16* lies between cosmids C43H8 and C35E7. The *daf-16* gene was identified by identifying deletions (*mgDf50*) and point mutations (*mg53* and *mg54*) within the forkhead gene on the cosmid R13H8 (Fig. 27). There are two major *daf-16* transcripts whose sequences are shown in Fig. 13A and Fig. 13B (SEQ ID NOS: 43 and \_\_, respectively). The amino acid sequences coding for the DAF-16 isoforms are shown in Figs. 14A-14C (SEQ ID NOS: 44-46).

We have detected three *daf-16* mutations: (1) a large deletion of conserved regions in *daf-16* (*mg ΔF50*) that proves that the *daf-16* null phenotype is a suppression of *daf-2* mutations; (2) an S to L substitution in exon 6 in *daf-16* (*mg 53*) that alters a conserved WKNSIRH motif; and (3) a nonsense mutation in exon 3 in *daf-16* (*mg 54*) that is predicted to truncate one of the *daf-16* differentially spliced isoforms. Interestingly, this spliced isoform has a distinct forkhead DNA binding domain and is therefore expected to bind to distinct promoters or combinatorial partners. This mutant is a weak suppressor of *daf-2*, suggesting that both DAF-16 isoforms are necessary for metabolic control.

Sequence analysis has revealed that DAF-16 is a member of the forkhead (FH) transcription factor family (Figs. 21A-21B). This strong amino acid homology indicates that DAF-16 is a transcription factor. Our genetic analysis

indicates that DAF-16 activity is regulated by the DAF-2/AGE-1 insulin signaling pathway. Precedent from another receptor kinase signaling pathway endorses this model:

the *C. elegans* LIN-31 forkhead protein has been shown to be regulated by a tyrosine kinase signaling cascade from the LET-23 EGF receptor homolog (Kim, *Genes Dev.* 7: 933-947, 1993). Consistent with a model that DAF-16 acts downstream of insulin signaling, forkhead transcription factors have also been implicated in metabolic regulation: another FH family member is mammalian HNF-3, an endoderm-specific transcription factor that acts at the same metabolic control protein promoters as HNF-1 and HNF-4, both of which are mutant in maturity onset diabetes of the young (MODY) (Yamagata et al., *Nature* 384: 455-458, 1996; Yamagata et al., *Nature* 384: 458-460, 1996).

The identification of DAF-16 as a forkhead transcription factor also explains much of the complex *daf* genetics of *C. elegans*. The convergence of DAF-7 TGF- $\beta$ -like signaling and DAF-2 insulin-like signaling is also explained by our discovery that DAF-16 is a FH protein and DAF-3 is a Smad protein: Precedent for an interaction between Smad and forkhead proteins has been found in *Xenopus*. Response to the TGF- $\beta$  superfamily relative activin in early frog development is mediated by an interaction between the distant relative of DAF-16 called FAST-1, and the Smad protein, Smad2 (*Nature* 383: 600-608, 1996). These proteins bind to an enhancer element that is very similar to the myosin II promoter to which DAF-3 binds (see below). Thus our molecular and genetic data indicate that the DAF Smad proteins and DAF-16 FH protein interact on metabolic control promoters.

Interestingly, analogously to *daf-16* bypass of the need for DAF-2 insulin receptor signaling in *daf-16* mutant animals, *lin-31* mutations suppress the need for LET-23 EGF signaling in *C. elegans* vulval development. These findings indicate

that the DAF-2 receptor, a downstream signaling molecule (AGE-1), and a transcription factor target DAF-16 are involved in insulin-like signaling in *C. elegans* development. Without being bound by any particular theory, we hypothesize that *C. elegans* insulin signaling via DAF-2 and AGE-1 activate DAF-16 transcriptional activity, so that in a *daf-2* or *age-1* mutant, or in dauer pheromone, DAF-16 acts as a repressor protein causing a metabolic shift to fat metabolism. Our analysis of *daf-16* expression shows that, like DAF-3, it is expressed in target tissues (Fig. 22). Our evidence indicates that Smad protein transcription factors (e.g., DAF 3, DAF8, DAF14) and DAF-16 act on a common set of promoters as combinatorial transcriptional regulators. Thus, it is at these metabolic genes that DAF-7 and TGF- $\beta$ -like and DAF-2 insulin-like signals converge to control metabolism. In addition, our evidence indicates that in the presence of DAF-2 signaling (mimicking high insulin), DAF-16 acts as an activator of transcription, causing a shift in metabolism toward glucose utilization for cell growth. The molecular analysis described herein suggests that lack of *daf-16* gene activity completely bypasses the need for insulin signaling in metabolic control by releasing metabolic control from DAF-16 repression. These data suggest that if a human DAF-16 homolog acts downstream of insulin signaling in humans, drugs could be developed that inhibit its activity to bypass the need for insulin signaling. Identification of a such a drug should provide a means for treating both Type I and Type II diabetes.

As shown in Figs. 21A-21B, the human FKHR and AFX genes, identified as oncogene breakpoints but not as insulin signaling genes, are much more closely related to DAF-16 than the next closest relative in either Genbank or in the 94% complete *C. elegans* genome sequence. These data indicate that FKHR and AFX are excellent candidates for subserving the same function as *C. elegans* DAF-16: transduction of insulin signals and convergence with DAF-7-like Smad signals.



Evidence for the *C. elegans* AKT kinase as the probable output of DAF-2/AGE-1 signaling.

We screened genetically for mutations that bypass the need for age-1 signaling. This was done by mutagenizing a strain carrying an age-1(mg44) null mutation (this mutation was heterozygous to allow the strain to grow). After two generations, animals that could survive without age-1 gene activity were selected by their lack of arrest at the dauer stage. We identified daf-16 mutations, as expected. However, we also identified two new gain of function mutations, sup(mg142) and sup(mg144).

sup(mg144) suppresses three different age-1 alleles, indicating that this mutation bypasses the need for AGE-1 production of PIP3. For example, sup(mg144) suppresses the dauer arrest of age-1(mg44), (m333), (mg109) such that fertile adults are formed. sup(mg144) does not suppress the lack of insulin signaling in the daf-2 mutant: daf-2(e1370); sup(mg144) form dauers at 25 degrees. This suggests that not all of the DAF-2 signaling output is via AGE-1. However, in the absence of both DAF-2 and AGE-1 signaling, sup(mg144) weakly suppresses, allowing some fertile adults to bypass arrest at the dauer stage. daf-2(e1370); sqt-1 age-1(mg44); sup(mg144) form 8% fertile adults, 12% sterile adults, and 80% dauers at 25 degrees.

Interestingly, sup(mg144) is a dominant suppressor of age-1 mutations. sqt-1 age-1(mg44); sup(mg144)/+ form 100% fertile adults. The sup(mg144) parental genotype does not affect this outcome. This data indicates that sup(mg144) is a dominant activating or dominant inactivating mutation.

Genetic mapping indicates that sup(mg144) may identify an activating mutation in the *C. elegans* AKT homologue (Fig. 25). By placing sup(mg144) in trans to a multiply marked chromosome (using PCR based RFLPs), we found that sup(mg144) maps to a 2 map unit genetic interval that includes *C. elegans* AKT

(Fig. 24).

2/39 sup(mg144 ) homozygous animals isolated from a sup(mg144)/polymorphic Bergerac chromosome parent recombined between sup(mg144)mg144 and stP6 (these animals also carried stP18). In this experiment mg144 was a het with RW7000 for three generations. So this places sup(mg144) approximately 2.2mu to the left of stP6).

1/39 sup(mg144 ) homozygous animals isolated from a sup(mg144)/polymorphic Bergerac chromosome parent recombined between sup(mg144) and bP1. In this experiment mg144 was a het with RW7000 for two generations. So this number is approximately 1/80 or 1.2 mu from bP1.

We generated a GFP fusion to AKT and showed that this gene is expressed at high levels in dauer larvae but at much lower levels and in fewer cells in wild type animals. (Figs. 26A-26B) Thus AKT represents a dauer regulated gene that may respond to DAF-16 and DAF-3 transcriptional control. Multiple probable binding sites, related to the DAF-3 binding site in myoII have been identified.

**sup(mg142) identifies another likely output of age-1 signaling**

mg142 suppresses three different age-1 alleles (age-1(mg44), age-1(m333), and age-1(mg109) at 20 degrees. age-1(mg44); sup(mg142 ) form fertile adults at 15 and 20. At 25 degrees, they form 33% fertile adults and 67% sterile adults.

sqt-1 age-1(mg44); mg142/+ form 14% fertile adults and 86% sterile adults when the parent was homozygous for mg142. sqt-1 age-1(mg44); mg142/+ form 67% fertile adults and 33% sterile adults when the parent was heterozygous for mg142. daf-2(e1370); mg142 form sterile adults at 25 degrees; daf-2(e1370); sqt-1 age-1(mg44); mg142 form sterile adults and dauers at 25 degrees.

Preliminary mapping places mg142 approximately 1.6mu left of unc-1 on LGX

### Diapause and Longevity

Weak *daf-2* and *age-1* mutants that do not arrest at the dauer stage nevertheless live much longer than wild-type (Larsen et al., *Genetics* 139: 1567-1583, 1995; Kenyon et al., *Nature* 366: 461-464, 1993; Dorman et al., *Genetics* 141: 1399-1406, 1995). This connection between longevity and diapause control may not be unique to *C. elegans*. Diapause arrest is an essential feature of many vertebrate and invertebrate life cycles, especially in regions with seasonal temperature and humidity extremes (Tauber et al., *Seasonal Adaptation of Insects*, Oxford University Press, New York, NY, 1986). Animals in diapause arrest slow their metabolism and their rates of aging, and can survive for periods for much longer than their reproductive lifespan (Tauber et al., *supra*, 1986).

Because insulin-like DAF-2/AGE-1 signaling mediates *C. elegans* diapause longevity control, the mammalian insulin signaling pathway may also control longevity homologously. In fact, the increase in longevity associated with decreased DAF-2 signaling is analogous to mammalian longevity increases associated with caloric restriction (Finch, *Longevity, Senescence and the Genome*, The University of Chicago Press, Chicago, 1990). It is possible that caloric restriction causes a decline in insulin signaling to induce a partial diapause state, like that induced in weak *daf-2* and *age-1* mutants. The induction of diapause-like states may affect post-reproductive longevity (Finch, *supra*), as in *C. elegans*. Alternatively, it is the changes in the mode and tempo of metabolism itself rather than diapause per se that causes increased longevity. Another long-lived *C. elegans* mutant, *clk-1*, may also regulate lifespan via such metabolic effects (Ewbank et al., *Science* 275: 980-983, 1997). This association of metabolic rate with longevity is also consistent with the correlation of free radical generation to aging (Finch, *supra*).

## **Synergistic Control of Metabolism and Diapause by Insulin and TGF- $\beta$**

### **Signaling Pathways**

In addition to DAF-2 signaling, the DAF-7 TGF- $\beta$  neuroendocrine signal is also necessary for reproductive development of *C. elegans* (Ren et al., *Science* 274: 1389-1391, 1996; Schackwitz et al., *Neuron* 17: 719-728, 1996). The signals in these two pathways are not redundant: animals missing either *daf-2* signaling or *daf-7* signaling (Fig. 3) shift their metabolism and arrest at the dauer stage (Table II). In addition the phenotypes caused by mutations in either pathway are strongly synergistic, suggesting that the two pathways are integrated.

Synchronised eggs were grown and counted as described above. *daf-1(m40)* and *daf-2(e1370)* form 100% dauer at 25°C. Numbers shown in Table II indicate percentage dauer formation and number of animals counted (in parenthesis). Data presented is the sum of three independent trials.

Table II. Synergy of *daf-1* and *daf-2*

	% dauer formation	
	15°C	20°C
<i>daf-1</i> (m40)	0.0 (532)	1.9 (909)
<i>daf-2</i> (e1370)	0.0 (798)	3.8 (503)
<i>daf-1</i> (m40); <i>daf-2</i> (e1370)	19.4 (747)	100 (718)

This data indicates that DAF-7 TGF- $\beta$  signals and DAF-2 ligand insulin-like signals are integrated. In support of this model, weak mutations in the *daf-2* insulin signaling pathway and in the *daf-7* TGF- $\beta$  signaling pathway are highly synergistic (Table II). Genetic epistasis analysis indicates that the DAF-7 and DAF-2 pathways are parallel rather than sequential (Vowels and Thomas, *Genetics* 130: 105-123, 1992; Gottlieb and Ruvkun, *Genetics* 137: 107-120, 1994). That is, *daf-16* mutations strongly suppress *daf-2* mutations but not *daf-7*, *daf-1*, or *daf-4* mutations, whereas *daf-3* mutations strongly suppress *daf-7*, *daf-1*, and *daf-4* mutations, but not *daf-2* mutations. Analogous synergism between activin and FGF tyrosine kinase pathways in *Xenopus* mesoderm induction has been noted (Green et al., *Cell* 71: 731-739, 1992).

A dauer-inducing pheromone regulates the production of DAF-7 by the ASI sensory neuron (Ren et al., *Science* 274: 1389-1391, 1996; Schackwitz et al., *Neuron* 17: 719-728, 1996). Because animals carrying *daf-7* nonsense or truncation mutations are responsive to pheromone (Golden and Riddle, *Proc. Natl. Acad. Sci. U.S.A.* 81: 819-823, 1984), we further suggest that the production of the insulin-like ligand for DAF-2 is also regulated by pheromone. It is not yet clear whether these DAF-7 and DAF-2 signals converge in target tissues or in other

regulatory (i.e., hormonal) cells; however the expression of the DAF-7 receptor pathway genes in essentially all target tissues (infra) suggests that integration occurs there.

### DAF-7 and Diabetes

Based on the data herein, we propose that in humans as in *C. elegans*, both a DAF-7-like neuroendocrine signal and insulin are necessary for metabolic control by insulin. According to this model, the failure of target tissues to respond to insulin signals in Type II diabetic patients could be due to defects either in the insulin or TGF- $\beta$ -like control pathways. Pedigree analysis has shown a strong genetic component in Type II diabetes (Kahn et al., *Annu. Rev. Med.* 47: 509-531,1996). In addition, obesity is also a major risk factor in Type II diabetes (Kahn et al., *Annu. Rev. Med.* 47: 509-531,1996). Genetic or obesity-induced (Hotamisligil et al., *Science* 259: 87-91, 1993; Lonnqvist et al., *Nat Med* 1: 950-953, 1995) declines in a DAF-7-like signaling pathway could underlie the lack of response to insulin in Type II diabetes, just as in *C. elegans* *daf-7* mutants cause metabolic defects very similar to *daf-2* mutants. The discovery that the DAF-7 and DAF-2 pathways converge indicates that DAF-7 hormonal signals are defective in diabetic conditions (for example, Type II diabetes), and that administration of human DAF-7 is useful for ameliorating the glucose intolerance, ketoacidosis, and atherosclerosis associated with diabetes. This is shown schematically in Figs. 17, 18, and 23.

Whereas the DAF-7 TGF- $\beta$  like and DAF-2 insulin-like signaling pathways converge to control diapause and metabolism, only the DAF-2/AGE-1 pathway has been implicated in reproductive adult stage longevity control in the absence of dauer formation (Larsen et al., *Genetics* 139: 1567-1583, 1995; Kenyon et al., *Nature* 366: 461-464, 1993; Dorman et al., *Genetics* 141: 1399-1406, 1995; and

Morris et al., *Nature* 382: 536-539, 1996). Both pathways control the longevity increase associated with dauer arrest, since dauer larvae live much longer than reproductive *C. elegans* (Riddle, In: *Caenorhabditis elegans* II, D. Riddle, T. Blumenthal, B. Meyer, J. Priess, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1997, pp. 739-768; Kenyon, op cit. pp., 791-813; Chayen and Bitensky, *Practical Histochemistry*, Chichester; New York: Wiley, 1991. The distinction between DAF-7 and DAF-2 regulation of longevity could also reflect a more profound regulation of metabolism by the DAF-2 pathway than the DAF-7 pathway (Fig. 4). For example, based on precedents from TGF- $\beta$  signaling in other systems and analysis of this pathway in *C. elegans*, all of the known signaling output of the DAF-7 TGF- $\beta$  pathway are via downstream Smad transcriptional regulation (infra). Insulin signaling, and by extension, DAF-2 signaling, is more ramified: outputs from this receptor regulate sugar transport, metabolic enzyme activities, translation of mRNAs encoding these and other enzymes, as well as transcription (White and Kahn, *J. Biol. Chem.* 269: 1-4, 1994). We suggest that it is the regulatory output distinct to the DAF-2 pathway that controls longevity. Alternatively, TGF- $\beta$  and insulin-like signals may converge only during the L1 stage, when diapause is regulated, and that after this stage, only DAF-2 signaling is necessary for normal metabolic control.

The involvement of insulin and TGF- $\beta$  signaling in *C. elegans* diapause control suggests that the homologous human pathways may similarly mediate response to famine. Just as environmental extremes can select for variation in the genetic pathways that regulate *C. elegans* dauer formation, famines and droughts in human history may have selected for analogous variants in the human homolog of the *daf* genes. In fact, heterozygous mice carrying either the *db* or *ob* recessive diabetes genes, survive fasting about 20% longer than wild type controls (Coleman, *Science* 203: 663-665, 1979). The high frequency of Type II diabetes

in many human populations may be the legacy of such selections.

**The DAF-3 Smad Protein Antagonizes DAF-7 TGF- $\beta$  Receptor Signaling in the *C. elegans* Dauer Regulatory Pathway**

In response to environmental signals *C. elegans* arrests development at the anatomically and metabolically distinctive third-larval dauer stage (Riddle In: C. elegans N, D.L. Riddle, T. Blumenthal, B.J. Meyer, J.R. Priess, eds., *Cold Spring Harbor Press*, 1997, pp. 739-768). Pheromone signal is transduced by chemosensory neurons (Bargmann and Horvitz, *Science* 251:1243, 1991) which couple to a TGF- $\beta$  signaling pathway (Ren et al., *Science* 274:1389, 1996; Schackwitz et al., *Neuron* 17:719, 1989), as well as an insulin-related signaling pathway (as discussed, *infra*) to trigger changes in the development of the many tissues remodeled in dauer larvae (Riddle, *supra*). Mutations in *daf-7* (a TGF- $\beta$  homolog (Estevez et al., *Nature* 365:644, 1993)), *daf-4* (a type II TGF- $\beta$  receptor (Estevez et al., *Nature* 365:644, 1993)), *daf-1* (a type I TGF- $\beta$  receptor), *daf-8*, and *daf-14* (Smad homolog) cause constitutive arrest at the dauer stage even in the absence of pheromone. These genes constitute a neuroendocrine signaling pathway that is active during non-dauer development: the DAF-7 TGF- $\beta$  signal is produced by the sensory neuron ASI during nondauer development, whereas *daf-7* expression in this neuron is inhibited during dauer-inducing conditions (Ren, *supra*).

*daf-7* and its receptors and Smad proteins are antagonists to *daf-3*. The dauer constitute phenotypes of mutations in the *daf-7* signal transduction pathway genes (including putative null mutations) are fully suppressed by mutations in *daf-3*. These genetic data indicate that in the absence of *daf-7* signaling, *daf-3* acts to induce dauer arrest.



To discern the molecular basis of the DAF-3 function in this pathway, we determined the sequence and expression pattern of *daf-3*. Cosmids in the *daf-3* genetic region were assayed for gene activity by transformation. Cosmid B0217 partially complemented a *daf-3* mutation, while other cosmids from the region did not (Fig. 5A). A subclone of B0217 containing only the Smad homolog, but no other coding regions also rescued *daf-3*. Our detection of mutations in the Smad homolog (see below) confirmed its assignment to *daf-3*. Analysis of *daf-3* cDNAs revealed that the gene was transcribed from fifteen exons and was alternatively spliced upstream of the region conserved in Smad proteins. (Fig. 5B) The biological activity of these alternatively spliced isoforms is unknown. The nucleotide (SEQ ID NO: 11) and amino acid sequences (SEQ ID NO: 12) of DAF-3 are shown in Figs. 11 and 12, respectively.

Thus far, the *C. elegans* DAF-3 Smad protein is most closely related in sequence to DPC4, which is a putative cofactor for Smad1, Smad2, and Smad3 (Zhang et al., *Nature*, 383:168, 1996; Lagna et al., *Nature*, 383:832, 1996; Savage et al., *Proc. Natl. Acad. Sci.*, 93:790, 1996; Hahn et al., *Science*, 271:350 (1996). Smads have two conserved domains (Wrana et al., *Trends Genet.*, 12:493, 1996). DAF-3 has these two domains; compared to its closest known relative DPC-4, *daf-3* has 55% amino acid identity in domain I and 30% in domain II (Fig. 5C). However, DPC-4 is not the mammalian DAF-3 homologue: *C. elegans* Sma-4, for example, is more closely related to DPC-4 than DAF-3.

We identified three mutations in *daf-3*, all of which were isolated as suppressors of *daf-7(e1372)*. *mgDf90* is a homozygous viable deletion of 15-90 kb that removes the entire Smad gene (Fig. 5A). *mgDf90* was identified as a spontaneous mutation that suppressed *daf-7* in the strain of GR1300 (*daf-7(e1372)* 111; *mut-6(st 702) unc-22 (St192) IV*). Thus, suppression of the *daf-7* dauer constitutive phenotype of *daf-3* is *daf-3* null phenotype, demonstrating that

wild-type DAF-3 acts antagonistically to signaling from the DAF-7 TGF- $\beta$  pathway signaling. *daf-3(mg125)* and *daf-3(mg132)* are missense mutations that alter conserved residues in domains 1 and 2 respectively (Fig. 5C). Most of the mutations detected in other Smads localize to a 45 amino acid segment of domain II (Wrana et al., *Trends in Genet.* 12:493, 1996). Clustering of mutations is observed even in DPC4, for which homozygous null mutations have been identified (Hahn et al., *Science* 271:350, 1996), so the clustering is unlikely to be due to selection for non-null mutations. This hotspot region was sequenced in nine *daf-3* alleles, and no mutations were detected. This difference in mutation location may be a simple statistical anomaly, or may indicate functional differences between DAF-3 and other Smad proteins, consistent with the fact that DAF-3 is antagonized, rather than activated, by an upstream TGF- $\beta$  molecule.

To determine where DAF-3 may function in control of dauer formation, we examined the expression pattern of a functional *daf-3*/Green Fluorescent Protein (GFP) fusion gene. This was accomplished by replacing a *AvrII*/*SacI* fragment from pGP8 with a PCR product in which several restriction sites were inserted after the last codon of *daf-3* before the stop codon. A GFP/*unc-54* 3' end PCR product from pPD95.81 was cloned into the 3' restriction sites to produce pGP19. This DAF-3/GFP fusion partially rescues a *daf-3* mutant (Fig. 7). GFP fluorescence therefore indicates the functional location of DAF-3. DAF-7 signaling from the ASI neuron begins during the L1 stage, and neuron ablations and dauer-formation assays in various environmental conditions indicate that the signal for dauer formation is also received during the first two larval stages (Ren et al., *Science* 274:1389, 1996; Schackwitz et al., *Neuron* 17:719, 1996; Bargmann and Horvitz, *Science* 251:1243, 1991; Golden and Riddle, *Developmental Biology* 102:368, 1984; Swanson and Riddle, *Developmental Biology* 84:27, 1981). Therefore, we most extensively examined L1 larvae.

Almost every transgenic animal showed strong *daf-3*/GFP expression in head neurons (Fig. 6A), the ventral nerve cord (both cell bodies and processes, see Fig. 6B), the intestinal cells (Fig. 6C), especially the membrane adjacent to the intestinal lumen, the tail hypodermis, and tail neurons. For all GFP scoring, animals were grown at 25-26°C. For scoring of DAF-3/GFP in wild-type and in dauer constitutive mutant backgrounds, three or more lines were scored in each case. A large number of animals were surveyed to determine the expression pattern, and at least 30 animals were scored head-to-tail, and expression was tallied for each tissue. About half of the transgenic animals have weak expression in V blast cells, P blast cells, hyp7 hypodermal cells, and the pharynx. The weak expression impedes cell identification, but the main body of the pharynx is filled, implying expression in pharyngeal muscle (Fig. 6A). Expression is rarely detected in dorsal body wall muscle. The expression pattern in older larvae and adults is similar to that of L1 animals. In addition, DAF-3/GFP is expressed in the distal tip cells and in their precursors, Z1.a and Z4.p, throughout development (Fig. 6D, Fig. 8). DAF-3/GFP is also strongly expressed in unidentified vulval cells. In wild-type embryos of 200-400 cells, DAF-3/GFP is expressed uniformly throughout the embryo (Fig. 6E). Under the conditions of the experiment, which promote reproductive growth, the subcellular localization of the DAF-3/GFP protein is mainly cytoplasmic (Fig. 6B-E, and see below).

Because DAF-3 activity may be regulated by the DAF-1 and DAF-4 TGF- $\beta$  receptors, we examined the expression of a DAF-4/GFP fusion in wild-type (Figs. 6A-6G). This construct complements a *daf-4* mutant. A 10 kb SalI fragment from cosmid CO5D2 contains 3 kb of sequence upstream of the *daf-4* transcriptional start, and all of the *daf-4* coding region except codons for the last fourteen residues of *daf-4*. This fragment was subcloned into the SalI site of the GFP plasmid TU#61 (Chalfie et al., *Science* 263: 802-805, 1994). This plasmid was injected

into the *daf-4(m72)* strain to test the fusion for DAF-4 activity. More than 95% of the transgenic animals were rescued for the dauer-constitutive and small phenotypes of *daf-4(m72)*, indicating that the fusion has robust DAF-4 activity. The pattern of DAF-4/GFP expression is similar to that of *daf-3/GFP*, except that DAF-4/GFP is localized to membranes, consistent with its role as a receptor. DAF-4/GFP is expressed more strongly in the pharynx (Figs. 6F-G), and more weakly in the ventral nerve cord cell bodies and the body hypodermis. Expression of DAF-4/GFP in wild-type animals is detected later than DAF-3/GFP. DAF-4/GFP is first detectable at late embryogenesis when the embryo resembles an L1 larva. The DAF-4/GFP construct contains an older version of GFP than in DAF-3/GFP; in the older version, the chromophore takes longer to mature. To verify that the difference in embryonic expression of DAF-4/GFP and DAF-3/GFP is not an artefact of the slower maturation time in the *daf-4* strain, we used anti-GFP antibodies to assay GFP. These antibodies should recognize the two forms of GFP equally well. We found that the antibodies recapitulated the results with direct GFP fluorescence: DAF-3/GFP is expressed in early embryos; DAF-4/GFP is not. DAF-4/GFP is also not expressed in membrane surrounding the intestinal lumen, unlike DAF-3/GFP.

The combination of the DAF-3 and DAF-4 expression patterns suggests that these genes act in target tissues to transduce pheromone-regulated DAF-7 neuroendocrine signals. The early expression of DAF-3 in embryos is also consistent with a model that DAF-3 acts during embryonic development, for example, to mediate the development of neuronal pathways that emit neuroendocrine signals that antagonize DAF-7 TGF- $\beta$  signaling during the L1 stage. However our data indicates that DAF-3 functions in transducing environmental signals during the L1 and L2 stages. This is supported by the following observations. (1) DAF-7 TGF- $\beta$  signal from ASI neurons occurs during

the L1 and L2 stages and is repressed by dauer-inducing environmental conditions.

(2) Expression of the DAF-4 type II receptor begins in very late embryogenesis.

(3) Expression patterns of DAF-3 and DAF-4 are coincident in most of the tissues remodeled during dauer morphogenesis. For example, the cuticle secreted by the hypodermis is modified, the pharynx is slimmed, and the lumen of the intestine is less convoluted. In addition, somatic gonad development is arrested in dauers, and the distal tip cell, in which DAF-3 is expressed, is an important regulator of that development (Kimble, *Developmental Biology* 87:286, 1981). In addition, the intestine and hypodermis of dauer larvae contain large fat stores indicative of a metabolic shift to fat storage. The expression of both the DAF-4 TGF- $\beta$  family receptor kinase and the DAF-3 Smad protein in these target tissues is consistent with a model that the DAF-7 neuroendocrine signal from the ASI neuron is received directly by these tissues during non dauer development. In addition, the observation that DAF-4 and DAF-3 are expressed in many of the same cells is consistent with a model that DAF-4 signaling to downstream Smads (DAF-8 and DAF-14 are likely candidates) directly regulates DAF-3 gene activity. The TGF- $\beta$  regulated nuclear localization and transcriptional activation of some Smad proteins suggests that DAF-3 might induce the dauer-specific changes by activating transcription in target tissues of genes required for dauer formation or repressing transcription of genes necessary for nondauer growth.

Smad1 and Smad2 relocate to become predominantly nuclear when the upstream TGF- $\beta$  signaling pathways are activated (Baker and Harland, *Genes and Development* 10: 1880, 1996; Hoodless et al., *Cell* 85:489, 1996; Liu et al., *Nature* 381:620, 1996; Macias-Silva et al., *Cell* 87:1215, 1996). In wild-type, DAF-3/GFP is primarily, although not exclusively, cytoplasmic. DAF-3/GFP subcellular distribution was examined in head neurons in the vicinity of ASI (the cell that produces the DAF-7 signal), as well as in intestinal cells. DAF-3/GFP

was predominantly cytoplasmic in all animals. However, in all animals, dim GFP fluorescence was observed in the nucleus of some of the cells with bright fluorescence, and in approximately twenty-five percent of the animals, equivalent DAF-3/GFP levels in the nucleus and cytoplasm has observed in one or more cells.

Because DAF-3 is antagonized by the other members of the DAF-7 TGF- $\beta$  pathway, we expect that DAF-3 is active (and perhaps localized to the nucleus) when these genes are inactive. We therefore observed the subcellular localization of the full-length DAF-3/GFP fusion protein in the head neurons, tail neurons, and intestine of dauer-constitutive mutant L1 worms, when DAF-3 gene activity is predicted to be highest. In DAF-1(*m402*), *daf-4(m72)*, *daf-7(m62)*, *daf-8(sa233)*, and *daf-14(m77)* mutants, DAF-3/GFP was predominantly cytoplasmic, although, as in wild-type, cells were seen with some GFP in the nucleus. In three *daf-4(m72)* mutant lines, DAF-3/GFP was localized to the nucleus more than in wild-type lines. When these strains were crossed to wild-type, the increased nuclear localization was seen in both the *daf-4* and wild-type segregants. Thus the increased nuclear GFP was a property of the array, rather than of *daf-4*. Even in the neurons nearest to ASI, where the DAF-7 signal should be strongest, no change in DAF-3/GFP subcellular localization was detected. The DAF-3/GFP fusion protein is predominantly cytoplasmic in L1 and L2 stages of larvae induced to form dauers by environmental conditions or by mutations in the insulin receptor pathway gene *daf-2*, rather than by mutations in the DAF-7 signaling pathway mutants (data not shown). The tissue-specific expression pattern of DAF-3/GFP was unaltered in these mutant backgrounds (data not shown).

The finding that DAF-3/GFP subcellular localization is not strongly responsive to DAF-7 signaling defects or to dauer-inducing environmental conditions does not rule out a role for DAF-3 in the nucleus in dauer formation. Even though we detect no change in DAF-3/GFP subcellular localization, we do

detect some DAF-3/GFP in nuclei, and a minor change in nuclear localization or a change in activity due to phosphorylation state may couple DAF-3 to DAF-7 signaling. In fact, the subcellular localization of *Drosophila* MAD protein is not detectably altered in wild-type when receptor signaling to MAD occurs; relocalization is seen only if the DPP ligand is drastically overexpressed. It is unlikely that a set of undiscovered TGF- $\beta$  receptors regulates DAF-3. The *C. elegans* genome sequence is 90% complete, and there is only one candidate TGF- $\beta$  receptor gene other than *daf-1* and *daf-4*. If this receptor were a positive regulator of DAF-3, mutants would be expected to, like *daf-3* mutants, suppress *daf-7* mutants. This receptor acts in a signaling pathway distinct from DAF-3, and it is not a suppressor of *daf-7*.

The implication from Smad homology that DAF-3 is active in the nucleus is supported by two additional observations. First, DAF-3/GFP is associated with chromosomes in intestinal cells during mitosis. These cells divide at the end of the L1 stage, and antibody staining with anti-GFP antibodies and anti- $\alpha$ -tubulin antibodies reveals that DAF-3/GFP is found associated with DNA between the spindles during mitosis (Fig. 8A). We see DAF-3 GFP co-localized with DAPI from prophase to late anaphase. DAF-3/GFP was associated with nuclei in prophase by the following criteria. The spindles were present on either side of the nucleus, but the nucleus has not completely broken down. In particular, an indistinct nucleolus was present. DAF-3/GFP continues to co-localize with DAPI until the chromosomes have separated to the normal distance by which nuclei are separated in the intestine, implying continued association until telophase. At this point in mitosis, DAF-3/GFP fades and becomes undetectable before the nuclei reform the nuclear envelope and nucleolus. Thus, DAF-3 can, indirectly or directly, bind DNA, consistent with the hypothesis that it is a transcriptional activator that acts in the nucleus. DAF-3 is not predicted from its mutant

phenotype to have a role in mitosis. It is possible that the brighter GFP on mitotic chromosomes is due to increased access to DNA due to the breakdown of the nuclear envelope. The second indication of DAF-3 function in the nucleus is our examination of a truncated DAF-3/GFP fusion that is missing most of conserved domain II. The truncated construct pGP7 consists of 8 kb of *daf-3* fused to GFP. An 8 kb EcoRI fragment from B0217 was cloned into the EcoRI site of pBluescript SK(-). A PvuII/SalI fragment of this subclone was ligated to a PvuII/SalI fragment from the GFP vector pPD95.81. The resulting plasmid contains ~2.5 kb of sequence upstream of the 5'-most exon of *daf-3* and coding region through the first 58 amino acid residues of domain II. The remaining 175 amino acids of *daf-3* and the 3' noncoding region are replaced with GFP and the *unc-54* 3' end. Three transgenic lines were isolated, and all had a similar phenotype. This fusion protein interferes with dauer induction; like a *daf-3* loss-of-function mutant, it suppresses mutations in *daf-7* (Fig. 7). This truncated protein is predominantly nuclear, suggesting that it represses dauer formation by acting in the nucleus (Fig. 8B). This result implies that wild-type DAF-3 also has a function in the nucleus. The full-length DAF-3/GFP construct also suppresses mutations in *daf-7*, as does a full-length DAF-3 construct without GFP (Fig. 7). This suppression indicates that overexpression of DAF-3 in the cytoplasm has dominant-negative activity, perhaps due to interference with DAF-3 interactions with receptors or cofactors such as other Smads.

The constitutive nuclear localization of truncated DAF-3/GFP fusion gene missing part of domain II suggests that control of Smad localization is complex. A Smad2 construct containing only the conserved domain II of the protein is constitutively nuclear, leading to the suggestion that the C-terminus is an effector domain, and the N-terminus tethers the protein in the cytoplasm (Baker and Harland, *Genes and Development* 10:1880, 1996; Hoodless et al., *Cell* 85:489,



1996; Liu et al., *Nature* 381:620, 1996; and Macias-Silva et al., *Cell* 87:1215, 1996). Our construct, in which the N-terminus is intact, is nuclear. Perhaps both domains provide tethering in the cytoplasm, and any disruption leads to nuclear entry. Alternatively, entry may be differently regulated for DAF-3 and Smad2. Significantly, Smad2, like Smad1 and Smad3 has an SSXS motif at the C terminus (Zhang et al., *Nature* 383:168, 1996; Lagna et al., *Nature* 383:832, 1996; Savage et al., *PNAS* 93:790; Baker and Harland, *Genes and Development* 10:1880, 1996; Hoodless et al., *Cell* 85:489, 1996; Liu et al., *Nature* 381:620, 1996; Macias-Silva et al., *Cell* 87:1215, 1996; and Graf et al., *Cell* 85:479, 1996); this motif is a substrate for phosphorylation and required for nuclear localization of Smad2 (Baker and Harland, *Genes and Development* 10:1880, 1996; Hoodless et al., *Cell* 85:489, 1996; Liu et al., *Nature* 381:620, 1996; and Macias-Silva et al., *Cell* 87:1215, 1996). DAF-3 has a single serine in the C terminal region, and DPC4 has no serines at this location.

We propose a model for the TGF- $\beta$  pathway in dauer formation (Figs. 9A-B). The DAF-7 TGF- $\beta$  ligand, which is produced by the ASI sensory neuron in conditions that induce reproductive organ (Ren et al., *Science* 274:1389, 1996; Schakwitz et al., *Neuron* 17:719, 1996), binds to the DAF-1/DAF-4 receptor kinases on target tissues. These receptor kinases then phosphorylate the Smads DAF-8 and/or DAF-14, analogous to the phosphorylation and activation of Smad1, Smad2, and Smad3 (Zhang et al., *Nature* 383:168, 1996; Lagna et al., *Nature* 383:832, 1996; Savage et al., *PNAS* 93:790, 1996). We propose that DAF-3 functions like its closest homolog, DPC4, which dimerizes with phosphorylated Smad1 and Smad2, even under conditions that do not lead to detectable DPC4 phosphorylation (Zhang et al., *Nature* 383:168, 1996; Lagna et al., *Nature* 383:832, 1996; and Savage et al., *PNAS* 93:790). We suggest that DAF-3 forms dauer-inducing homodimers in the absence of DAF-7 signaling (Figs. 9A-B) that

The dauer genetic pathway represents a neuroendocrine pathway for control of a diapause arrest and its associated shifts in metabolism and rates of senescence (Ren et al., *Science* 274:1389, 1996; Schackwitz et al., *Neuron* 17:719, 1996; and Georgi et al., *Cell* 61:635, 1990). Similarly, activins, members of the TGF- $\beta$  family, were originally identified based on their neuroendocrine regulatory activity, for example, in regulation of gonadotropin signaling (Vale et al., in *Peptide Growth Factors and Their Receptors*, Sporn and Roberts, Eds., Springer-Verlag, Heidelberg, 1990). The DAF-7 signal is not the only signal that is necessary for reproductive development. Because mutations in the DAF-7 TGF- $\beta$  pathway and in the DAF-2 insulin-like signaling pathway cause the same dauer arrest phenotypes, we propose that both the DAF-7 TGF- $\beta$  signals and the DAF-2 insulin-like signals are necessary for reproductive development. The involvement of an insulin-like signaling pathway in diapause with its associated metabolic shifts is consistent with metabolic regulation by insulin in vertebrates. Genetic experiments indicate that these pathways act in parallel (Riddle et al., *Nature* 290:668, 1981; Vowels and Thomas, *Genetics* 130:105, 1992; and Thomas et al.,

*Genetics* 134:1105, 1993). In particular, *daf-3* mutants efficiently suppress *daf-7* mutants, but not *daf-2* mutants, and *daf-16* mutants efficiently suppress *daf-2* mutants, but poorly suppress *daf-7* mutants. It is not yet clear whether these two signaling pathways coverage on target tissues or in other regulatory (e.g., hormone secreting) cells. However, the expression of the DAF-7 receptor pathway genes and the DAF-16 gene in essentially all target tissues suggests that the TGF- $\beta$  and insulin pathways act there, and therefore that integration must occur there. Thus, we suggest in Figs. 9A and 9B that the DAF-2 pathway converges on DAF-3/DAF-8/DAF-1 Smad signaling to regulate metabolic gene expression in target tissues.

The integration of insulin-like and TGF- $\beta$  signals in metabolic control has important implications for the molecular basis of diabetes. For example, these converging pathways for dauer control suggest that in human metabolic control both a DAF-7-like signal and insulin may be necessary for full metabolic control. Thus, declines in signaling from the human homolog of DAF-7 could underlie the insulin resistance associated with Type II diabetes. In fact the dauer pheromone has been reported to be a fatty acid and to cause down-regulation of DAF-7 expression (Ren et al., supra). Thus pheromone regulation of metabolism may be related to mammalian obesity induced diabetes, and a human mutation in DAF-7 or its receptors is expected to contribute to a diabetic condition, just like mutations in the insulin receptor. In addition if obesity or age or both cause human DAF-7 to decline, e.g., under high leptin conditions, such a result would explain late onset/obesity related diabetes.

### **Cloning Mammalian DAF Sequences**

Based on our isolation of novel nematode DAF cDNAs, the isolation of mammalian DAF nucleic acid sequences, including human DAF sequences, is

made possible using the sequences described herein and standard techniques. In particular, using all or a portion of a nematode DAF sequence, one may readily design oligonucleotide probes, including degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either strand of the DNA.

Exemplary probes or primers for isolating mammalian DAF sequences preferably correspond to conserved blocks of amino acids, for example, conserved DAF motifs. Exemplary motifs are as follows:

DAF-2 (tyrosine kinase domain) (SEQ ID NO: 33)

1242 KFHEWAAQICDGMAYLESCLKFCHRDLAARNCMINRDETVKIGDFGM  
ARDLFYHDYYKPSGKRMMPVRWMSPELKDGGKFDSDVWSFGVVLYE  
MVTLGAQPYIGLSNDEVNLNYIGMARKVIKKPEC 1368

DAF-2 (ligand binding domain) (SEQ ID NO: 34)

242 NTTTCQKSCAYDRLLPTKEIGPGCDANGDRCHDQCVGGCERVNDATA  
CHACKNVYHKGKCKIEKCAHLYLLLQRRCVTREQLQLNPVLSNKTVPK  
ATAGLCSDKCPDGYQINPDDHRECRKCVGKCEIVC 372

DAF-2 (67 amino acid motif) (SEQ ID NO: 79)

1158 AIKINVDDPASTENLNYLMEANIMKNFKTNFIVQLYGVISTVQPAMV  
VMEMMDLGNLRDYLRSKRED 1224

DAF-2 (54 amino acid motif) (SEQ ID NO: 80)

1362 VIKKPECCENYWKVMKMCWRYSPRDRPTFLQLVHLLAAEASPEFR  
DLSFVLTD 1415

DAF-2 (69 amino acid motif) (SEQ ID NO: 81)

404 KQDSGMASELKDIFANIHTITGYLLVRQSSPFISLNMFRNLRRIEAKSL  
FRNLYAITVFENPNLKKLFD 472

DAF-2 (52 amino acid motif) (SEQ ID NO: 82)

98 FPHLREITGTLLVFETEGLVDLRKIFPNLRVIGGRSLIQHYALIIYRN  
PDLE 149

DAF-2 (46 amino acid motif) (SEQ ID NO: 83)

149 EIGLDKLSVIRNGGVRIIDNRKLCYTKTIDWKHLITSSINDVVVDN 194

DAF-2 (36 amino acid motif) (SEQ ID NO: 84)

1112 YNADDWELRQDDVVLGQQCGEGSFGKVYLGTGNNVV 1147

DAF-3 (Smad Domain I) (SEQ ID NO: 35)

240 FDQKACESLVKKLKDKKNDLQNLIDVVLSSKGTKYTGCITIPRTL DGR  
LQVHGRKGFPHVVY GKLWRFNEMTKNETRHVDHCKHAFEMKSDMVC  
VNPYHYEIVI 342

DAF-3 (Smad Domain II) (SEQ ID NO: 36)

690 NRYS LGLEPNPIREPVAFKVRKAIVD GIRFSYKKDGSVWLQNRMKYPV  
FVTSGYLDEQSGGLKKDKVHKVYGCASIKTF 768

DAF-3 (79 amino acid motif) (SEQ ID NO: 85)

819 DSLAKYCCVRVSFCKGFGEAYPER 842

DAF-16 (forkhead DNA binding domain) (SEQ ID NO: 37)

727 KKTTTTRRNAWGNMSY AELITTAIMASPEKRLTLAQVYEW MVQNPY  
FRDKGDSNSSAGWKNSIRHNLSLHSR FMRIQNEGAGKSSWWVINPDAKPG  
MNPRRTRERS 1044

DAF-16 (103 amino acid motif) (SEQ ID NO: 54)

242 KKTTTTRRNAWGNMSY AELITTAIMASPEKRLTLAQVYEW MVQNPY  
FRDKGDSNSSAGWKNSIRHNLSLHSR FMRIQNEGAGKSSWWVINPDAKPG  
MNPRRTR 344

DAF-16 (41 amino acid motif) (SEQ ID NO: 55)

137 TFMNTPDDVMMNDDMEPIPRDRCNTWPMRRPQLEPPLNSSP 177

DAF-16 (109 amino acid motif) (SEQ ID NO: 56)

236 DDTVSGKKTTTTRRNAWGNMSY AELITTAIMASPEKRLTLAQVYEW  
VQNPYPYFRDKGDSNSSAGWKNSIRHNLSLHSR FMRIQNEGAGKSSWWVI  
NPDAKPGMNPRRTR 344

DAF-16 (98 amino acid motif) (SEQ ID NO: 58)

372 KPNPWGEESYSDIIAKALESAPDGR LKLNEIYQWFSDNIPYFGERS SPE  
EAAGWKNSIRHNLSLHSR FMRIQNEGAGKSSWWVINPDAKPGMNP  
RRTR 469

Using such motifs, mammalian DAF-2, DAF-3, and DAF-16 genes may be isolated from sequence databases (for example, by the use of standard programs such as Pileup). Alternatively, such sequences may be used to design degenerate oligonucleotide probes to probe large genomic or cDNA libraries directly. General methods for designing and preparing such probes are provided, for example, in

Ausubel et al., *Current Protocols in Molecular Biology*, 1996, Wiley & Sons, New York, NY; and *Guide to Molecular Cloning Techniques*, 1987, S. L. Berger and A. R. Kimmel, eds., Academic Press, New York. These oligonucleotides are useful for DAF gene isolation, either through their use as probes for hybridizing to DAF complementary sequences or as primers for various polymerase chain reaction (PCR) cloning strategies. If a PCR approach is utilized, the primers are optionally designed to allow cloning of the amplified product into a suitable vector. PCR is particularly useful for screening cDNA libraries from rare tissue types.

Hybridization techniques and procedures are well known to those skilled in the art and are described, for example, in Ausubel et al., *supra*, and *Guide to Molecular Cloning Techniques, supra*. If desired, a combination of different oligonucleotide probes may be used for the screening of the recombinant DNA library. The oligonucleotides are, for example, labelled with  $^{32}\text{P}$  using methods known in the art, and the detectably-labelled oligonucleotides are used to probe filter replicas from a recombinant DNA library. Recombinant DNA libraries (for example, human cDNA libraries, such as hypothalamus- or pancreas-derived cDNA libraries, particularly for DAF-2 and DAF-7 cDNAs) may be prepared according to methods well known in the art, for example, as described in Ausubel et al., *supra*, or may be obtained from commercial sources.

For detection or isolation of closely related DAF sequences, high stringency hybridization conditions may be employed; such conditions include hybridization at about 42°C and about 50% formamide; a first wash at about 65°C, about 2X SSC, and 1% SDS; followed by a second wash at about 65°C and about 0.1% SDS, 1X SSC. Lower stringency conditions for detecting DAF genes having less sequence identity to the nematode DAF genes described herein include, for example, hybridization at about 42°C in the absence of formamide; a first wash at

about 42°C, about 6X SSC, and about 1% SDS; and a second wash at about 50°C, about 6X SSC, and about 1% SDS.

As discussed above, DAF-specific oligonucleotides may also be used as primers in PCR cloning strategies. Such PCR methods are well known in the art and are described, for example, in *PCR Technology*, H.A. Erlich, ed., Stockton Press, London, 1989; *PCR Protocols: A Guide to Methods and Applications*, M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, eds., Academic Press, Inc., New York, 1990; and Ausubel et al., *supra*. Again, sequences corresponding to conserved regions in a DAF sequence (for example, those regions described above) are preferred for use in isolating mammalian DAF sequences. Such probes may be used to screen cDNA as well as genomic DNA libraries.

Sequences obtained are then examined (for example, using the Pileup program) to identify those sequences having the highest amino acid sequence identity to the *C. elegans* sequence, particularly in or between conserved DAF domains (for example, those domains described above). In one particular example, the human FKHR and AFX genes are 10<sup>33</sup> more closely related to the DAF-16 forkhead domain than the next most closely related forkhead domain protein, making FKHR and AFX candidates for mammalian DAF-16 genes.

Following isolation of such candidate genes by sequence homology, the genes are then tested for their ability to functionally complement a *daf* mutation. This is most readily assayed by transformation of the sequence into a *C. elegans* strain having an appropriate mutant background. Exemplary *C. elegans* transformation techniques are described, for example, in Mello et al., *EMBO J.* 10: 3959-3970, 1991, and assays for DAF-2, DAF-3, and DAF-16 polypeptide function are described herein. To be considered useful in the invention, a mammalian sequence need not fully complement a *C. elegans* defect, but must provide a detectable level of functional complementation.



For example, addition of DAF-7, TGF- $\beta$ -like growth factor to an insulin responsive cell line (e.g., the 3T3-L1 cell line) may accentuate insulin responsiveness. Similarly genetic transformation of such a cell line with wild type or dominantly activated versions of a DAF, AGE, or AKT gene may alter metabolism. Such perturbations of metabolic control are stringent tests of candidate genes as DAF, AGE, or AKT homologues.

In addition, if that mammalian candidate homologue acts in a metabolic control pathway, and is expressed in similar metabolic control tissues (liver, adipose), it is likely to function homologously to DAF proteins from *C. elegans*. Addition of a wild type or activated DAF, AKT, or AGE protein (for example by VP16 activation of the DAF-3 or DAF-16 transcription factors) can confer on cell lines altered metabolic phenotypes. Thus supplying *daf*, *age*, or *akt* gene activity to such a cell line can alter its metabolism. This is one exemplary test of homologous DAF function in metabolic control.

### DAF Polypeptide Expression

In general, DAF polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of DAF-encoding cDNA fragment (e.g., one of the cDNAs described herein or isolated as described above) in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The DAF polypeptide may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae*, insect cells, e.g., Sf9 or Sf21 cells, or

mammalian cells, e.g., COS 1, NIH 3T3, or HeLa cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, see, e.g., Ausubel et al., *supra*). The method of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*supra*); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P.H. Pouwels et al., 1985, Supp. 1987).

One preferred expression system is the baculovirus system (using, for example, Sf9 cells and the method of Ausubel et al., *supra*). Another baculovirus system makes use of the vector pBacPAK9 and is available from Clontech (Palo Alto, CA).

Alternatively, an DAF polypeptide is produced in a mammalian system, for example, by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (*supra*); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (*supra*). In one example, cDNA encoding the DAF protein is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the DAF protein-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300  $\mu$ M methotrexate in the cell culture medium (as described in Ausubel et al., *supra*). This dominant selection may be accomplished in most cell types. Recombinant protein expression may be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (*supra*); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for

this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., *supra*). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR<sup>-</sup> cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

In yet other alternative approaches, the DAF polypeptide is produced *in vivo* or, preferably, *in vitro* using a T7 system (see, for example, Ausubel et al., *supra*, or other standard techniques).

Once the recombinant DAF protein is expressed, it is isolated, e.g., using affinity chromatography. In one example, an anti-DAF protein antibody (e.g., produced as described herein) may be attached to a column and used to isolate the DAF protein. Lysis and fractionation of DAF protein-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., *supra*).

Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, 1980).

Polypeptides of the invention, particularly short DAF polypeptide fragments, may also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

These general techniques of polypeptide expression and purification may also be used to produce and isolate useful DAF fragments or analogs (described herein).

### Anti-DAF Antibodies

Using any of the DAF polypeptides described herein or isolated as described above, anti-DAF antibodies may be produced by any standard technique. In one particular example, a DAF cDNA or cDNA fragment encoding a conserved DAF domain is fused to GST, and the fusion protein produced in *E. coli* by standard techniques. The fusion protein is then purified on a glutathione column, also by standard techniques, and is used to immunize rabbits. The antisera obtained is then itself purified on a GST-DAF affinity column, for example, by the method of Finney and Ruvkun (*Cell* 63:895-905, 1990), and is shown to specifically identify GST-DAF, for example, by Western blotting.

Polypeptides for antibody production may be produced by recombinant or peptide synthetic techniques (see, e.g., *Solid Phase Peptide Synthesis*, *supra*; Ausubel et al., *supra*).

For polyclonal antisera, the peptides may, if desired, be coupled to a carrier protein, such as KLH as described in Ausubel et al, *supra*. The KLH-peptide is mixed with Freund's adjuvant and injected into guinea pigs, rats, or preferably rabbits. Antibodies may be purified by any method of peptide antigen affinity chromatography.

Alternatively, monoclonal antibodies may be prepared using a DAF polypeptide (or immunogenic fragment or analog) and standard hybridoma technology (see, e.g., Kohler et al., *Nature* 256:495, 1975; Kohler et al., *Eur. J. Immunol.* 6:511, 1976; Kohler et al., *Eur. J. Immunol.* 6:292, 1976; Hammerling et al., *In Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, NY, 1981; Ausubel et al., *supra*).

Once produced, polyclonal or monoclonal antibodies are tested for specific DAF recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., *supra*). Antibodies which specifically recognize a

DAF polypeptide described herein are considered to be useful in the invention. Anti-DAF antibodies, as isolated above, may be used, e.g., in an immunoassay to measure or monitor the level of DAF polypeptide produced by a mammal or to screen for compounds which modulate DAF polypeptide production (for example, in the screens described herein). In one particular example, antibodies to human DAF-7 polypeptide are useful for screening blood samples from patients to determine whether they possess decreased DAF-7 polypeptide levels. Such antibodies may be used in any immunological assay, for example, an ELISA assay, and a decrease in DAF-7 is taken as an indication of a diabetic condition, for example, obesity onset Type II diabetes. In another particular example, anti-DAF antibodies are useful for carrying out pedigree analysis. For example, blood samples from individuals may be screened with anti-DAF-7 antibodies to detect those members of a family with a predisposition to a diabetic condition. Anti-DAF antibodies may also be used to identify cells that express a DAF gene.

#### *DAF-7 therapy for obesity-onset Type II diabetes*

Our data indicates that DAF-7 represents an endocrine hormone for metabolic control that acts synergistically with insulin. Declines in DAF-7 may be induced by obesity, just as the dauer pheromone, a fatty acid, causes declines in *C. elegans* DAF-7 production.

Accordingly, obesity onset Type II diabetes, glucose intolerance, and the associated atherosclerosis may be treated if DAF-7 hormone is injected intramuscularly or intravenously (Fig. 23).

In addition, antibodies to human DAF-7 should detect declines in DAF-7 in pre-diabetic, glucose-intolerant, or obesity induced diabetes. Such antibodies will detect DAF-7 levels in blood, just as insulin levels are detected in metabolic disease.

DAF-7 therapeutic potential and dosage can be developed in mouse models of obesity onset diabetes - the db and ob mouse.

DAF-7 will be injected either intravenously or intramuscularly, in analogy to insulin therapy.

The decision of which classes of diabetics to treat with DAF-7 will come from a combination of blood tests for DAF-7 levels and genetic testing for which *daf*, *age*, or *akt* mutations a particular diabetic, pre-diabetic patient carries.

### **Screening Systems for Identifying Therapeutics**

Based on our experimental results, we have developed a number of screening procedures for identifying therapeutic compounds (e.g., anti-diabetic and anti-obesity pharmaceuticals or both) which can be used in human patients. In particular examples, compounds that down regulate *daf-3* or *daf-16* or their human homologs are considered useful in the invention. Similarly, compounds that up regulate or activate *daf-1*, *daf-2*, *daf-4*, *daf-7*, *daf-8*, *daf-11*, *daf-14*, *age-1*, and *akt* (or each of their corresponding human homologs) are also considered useful as drugs for the treatment of impaired glucose tolerance conditions, such as diabetes and obesity. In general, the screening methods of the invention involve screening any number of compounds for therapeutically active agents by employing any number of *in vitro* or *in vivo* experimental systems. Exemplary methods useful for the identification of such compounds are detailed below.

The methods of the invention simplify the evaluation, identification, and development of active agents for the treatment and prevention of impaired glucose tolerance conditions, such as diabetes and obesity. In general, the screening methods provide a facile means for selecting natural product extracts or compounds of interest from a large population which are further evaluated and condensed to a few active and selective materials. Constituents of this pool are



desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their anti-diabetic and anti-obesity activities should be employed whenever possible.

When a crude extract is found to have anti-diabetic or anti-obesity activities or both, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having anti-diabetic or anti-obesity activities. The same *in vivo* and *in vitro* assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for the treatment of pathogenicity are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value are subsequently analyzed using any standard animal model of diabetes or obesity known in the art.

There now follow examples of high-throughput systems useful for evaluating the efficacy of a molecule or compound in treating (or preventing) an impaired glucose tolerance condition.



### Nematode Release of Dauer Arrest Bioassays

To enable mass screening of large quantities of natural products, extracts, or test compounds in an efficient and systematic fashion, *C. elegans* mutant dauer larvae (e.g., *C. elegans* containing mutations described herein, such as *C. elegans daf-2* mutant dauer larvae) are cultured in wells of a microtiter plate, facilitating the semiautomation of manipulations and full automation of data collection. As discussed above, compounds that down regulate DAF-3 or DAF-16 activities or up regulate DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT activities are considered useful in the invention. Such compounds are identified by their effect on dauer formation in *C. elegans* strains carrying mutations in these genes (as described above).

In particular examples, nematodes bearing mutations in the DAF-2 polypeptide arrest as dauer larvae, never producing progeny. All of the metabolic and growth arrest phenotypes caused by lack of *daf-2* are suppressed by mutations in *daf-16*. Mutations in the PI 3-kinase, AGE-1, have the same phenotype as lack of *daf-2*, and such mutations are also suppressed by *daf-16* mutations. Biochemical analysis of insulin signaling in mammals supports the view that AGE-1 transduces signals from the DAF-2 receptor by generating a PIP3 signal. Because *daf-16* mutations suppress lack of *daf-2*, or *age-1* gene activity, it is believed that PIP3 down regulates or modifies *daf-16* gene activity. The biochemical overlap between DAF-2/AGE-1 and insulin receptors/PI 3-kinase indicates that the human homolog of the *C. elegans daf-16* gene acts in the insulin pathway as well. Thus, the *C. elegans* insulin signaling pathway yields the surprising result that the animals can live without insulin signaling, provided they are mutant in *daf-16*. This analysis therefore indicates that a compound that inhibits DAF-16 activity would reverse the effects of diabetic lesions, e.g., in the production or secretion of insulin or in the reception of insulin signals by target

tissues. Such drugs would be expected to be efficacious in the treatment of insulin deficiencies due to pancreatic  $\beta$  cell destruction in Type I diabetes, as well as some Type II diabetes due to defects in insulin signaling.

To evaluate the ability of a test compound or an extract to decrease *daf-16* gene activity, mutant *daf-2* (e1370); *daf-16* (mgDf50) animals carrying an integrated human DAF-16 gene are incubated in microtiter dishes in the presence of a test compound. This human DAF-16 gene supplies all of the DAF-16 activity in the *C. elegans* strain and thus allows *daf-2*-induced dauer arrest unless its activity is decreased by the candidate test compound. If desired, various concentrations of the test compound or extract can be inoculated to assess the dosage effect. Control wells are incubated in the absence of a test compound or extract. Plates are then incubated at 25°C. After an appropriate period of time, e.g., 2 to 5 days, wells are examined for progeny. The presence of progeny is taken as an indication that the test compound or extract is effective at inhibiting *daf-3* or *daf-16* activity, and therefore is considered useful in the invention. Any compound that inhibits DAF-16 gene activity (or activates upstream signaling in the absence of receptor function) will allow reproduction. This is shown schematically in Fig. 19.

Alternatively, a diabetic condition may arise from defects in the DAF-7 TGF- $\beta$  signaling pathway. Since a decrease in DAF-3 activity bypasses the need for DAF-7 activity in *C. elegans* metabolic control, drugs that down regulate DAF-3 activity are useful for ameliorating the metabolic defects associated with diabetes. To screen for such drugs, *daf-7* (e1372); *daf-3* (mg90) nematodes expressing human DAF-3 are exposed to chemicals as described above. In this strain, human DAF-3 supplies all DAF-3 activity, causing *daf-7* induced dauer arrest unless its activity is inhibited (Fig. 20). Compounds capable of inhibiting this activity are considered useful therapeutics in the invention.

Finally, in a less complex screen for drugs that inhibit *C. elegans* *daf-3* or *daf-16*, *daf-7* or *daf-2* mutants are directly screened for compounds that decrease *C. elegans* *daf-3* or *daf-16* gene activity.

In addition, *C. elegans* worms carrying other *daf* mutations may be utilized in an assay to obtain additional information on the mode of action of the test compound in the insulin or TGF- $\beta$  signaling pathways. For example, a drug having PIP3 agonist activity would be expected to allow *age-1* and *daf-2* mutants (but not *akt* or *daf-7* mutants) to not arrest at the dauer stage. Similarly, drugs that inhibit *daf-3* are expected to suppress *daf-7* mutants but not *daf-2* or *age-1* mutants.

### Other Screening Assays

Other drug screening assays may also be performed using either *C. elegans* worms or mammalian cell cultures. If desired, such assays may include the use of reporter gene constructs.

For example, evaluation of the effects of test compounds on dauer formation or reporter gene expression in mutant *C. elegans* strains expressing particular human homologs of the *daf*, *age*, or *akt* genes (i.e., humanized *C. elegans*) represent useful screening methods. Expression of the human homologs in *C. elegans* is accomplished according to standard methods and, if desired, such genes may be operatively linked to a gene promoter obtained from *C. elegans*. Such promoters include, without limitation, the *C. elegans* *daf-16*, *age-1*, *daf-3*, *daf-4*, and *akt* gene promoters. For example, the 2.5 kb *age-1* promoter can be generated and isolated by employing standard PCR methods using the following primers: 5'GGAAATATTTTAGGCCAGATGCG3' (SEQ IS NO: 49) and 5'CGGACAGTCCTGAATACACC3' (SEQ ID NO: 50).

Additionally, mammalian tissue culture cells expressing *C. elegans* *daf*, *age-1*, or *akt* homologs may be used to evaluate the ability of a test compound or extract to modulate the insulin or TGF- $\beta$  signaling pathways. Because the signaling pathways from the ligands, receptors, kinase cascades, and downstream transcription factors are conserved from man to worm, test compounds or extracts that inhibit or activate the worm signaling proteins should also inhibit or activate their respective human homolog. For example, our identification that DAF-16 is a transcription factor that acts downstream of insulin-like signaling in *C. elegans* indicates that human DAF-16 transcription reporter genes also can be used to identify drugs that inhibit any of the kinases in the signaling pathway downstream of insulin signaling. For example, the use of DAF-16 and DAF-3 protein binding sites in reporter gene constructs may be used to monitor insulin signaling. Candidate compounds mimicking insulin signaling (e.g., PIP3 agonists) are expected to increase reporter gene expression and are considered useful in the invention.

#### **Reporter Gene Construct**

In one particular example, the invention involves the use of a reporter gene that is expressed under the control of a *C. elegans* gene promoter, e.g., a promoter that includes the TCTCGTTGTTTGCCGTCGGATGTCTGCC (SEQ ID NO: 51) enhancer element, such as the *C. elegans* pharyngeal myosin promoter (Okkema and Fire, *Development* 120: 2175-2186, 1994). This enhancer element is known to respond to DAF-3 regulation (i.e., in *daf-7* mutants, where *daf-3* is active, the element confers high level expression to reporter genes; whereas in a *daf-7*; *daf-3* mutant (for example, *daf-7* (e1372); *daf-3*), the element confers low level expression to reporter genes). Other equivalent enhancer elements may also be used in the invention, e.g., the enhancer element which is bound by the *Xenopus*

Smad1 and Fast1 forkhead proteins (*Nature* 383 600-608, 1996). The enhancer element is cloned upstream of any standard reporter gene, e.g., the luciferase or green fluorescent protein (GFP) reporter genes. In preferred embodiments, the GFP reporter gene is used in *C. elegans*. In other preferred embodiments, either the GFP or the luciferase reporter genes may be used in a mammalian cell based assay. The reporter gene construct is subsequently introduced into an appropriate host (e.g., *C. elegans* or a mammalian cell) according to any standard method known in the art. Analysis of reporter gene activity in the host organism or cell is determined according to any standard method, e.g., those methods described herein. Such reporter gene (and host cell systems) are useful for screening for drugs that modulate insulin or DAF-7 metabolic control signaling.

### *C. elegans*

In one working example, the above-described reporter gene construct is introduced into wild-type *C. elegans* according to standard methods known in the art. If the enhancer element is operational, then it is expected that reporter gene expression is turned on. Alternatively, in *daf* mutants (e.g., *daf-7* or *daf-2* mutants, where insulin signaling is defective) carrying the above-described reporter gene construct, reporter gene activity is turned off.

Using this on/off distinction, test compounds or extracts are evaluated for the ability to disrupt the signaling pathways described herein. In one working example, *daf-2* mutant worms carrying the reporter gene construct are used to assay the expression of the reporter gene. The majority of worms expressing the reporter gene will arrest at the dauer stage because of the *daf-2* phenotype. If however the test compound or extract inhibits DAF-16 activity, then the worms will exhibit a *daf-2*; *daf-16* phenotype (i.e., do not arrest), developing to produce eggs. Such eggs are selected using a bleach treatment and reporter gene

expression in the test compound or extract is assayed according to standard methods, e.g., worms are examined with an automated fluorometer to reveal the presence of reporter gene expression, e.g., GFP. Candidate compounds that suppress the *daf-2* phenotype or turn on reporter gene expression, i.e., activate signals in the absence of DAF-2 receptor (e.g., PIP3 mimetics) or inactivate DAF-16, are considered useful in the invention.

Analogous screens may also be performed using *daf-7* mutants as a means to identify drugs that inactivate other *daf*-genes, such as DAF-3, or compounds that activate the DAF-1/DAF-4 receptors. Such screens may be coupled to reporter screens, for example, using GFP reporter genes whose expression is under DAF-3 transcriptional control (e.g., the *myoII* element). Drugs identified in such screens are useful as DAF-7 mimetics. Because DAF-7 expression may be down regulated in obesity, such drugs are useful in the treatment of obesity induced Type II diabetes

In yet another working example, *C. elegans* DAF-3 and DAF-16 genes can be replaced with a human homolog, (e.g., FKHR for DAF-16), and screens similar to those described above performed in the nematode system. Because drugs may act upstream of the transcription factors, it is useful to replace DAF-1, DAF-4, DAF-8, DAF-14, DAF-2, DAF-3, DAF-16, or AGE-1 with the appropriate human homolog, and to screen the humanized *C. elegans* animals. Such screens are useful for identifying compounds having activities in humans.

### **Mammalian Cells**

Mammalian insulin-responsive cell lines are also useful in the screening methods of the invention. Here reporter gene constructs (for example, those described above) are introduced into the cell line to evaluate the ability of a test compound or extract to modulate insulin and TGF- $\beta$  signaling pathways using a

second construct expressing a *C. elegans daf*, *age*, or *akt* gene or their corresponding human homologs. Exemplary cell lines include, but are not limited to, mouse 3T3, L6, and L1 cells (MacDougald et al., *Ann. Rev. Biochem.* 64: 345-373, 1995). Introduction of the constructs into such cell lines is carried out according to standard methods well known in the art.

To test a compound or extract, it is added to the cell line, and reporter gene expression is monitored. Compounds that induce reporter gene expression in the absence of insulin or DAF-7 signaling are considered useful in the invention. Such compounds may also turn the cells into adipocytes, as insulin does.

Compounds identified in mammalian cells may be tested in other screening assays described herein, and, in general, test compounds may be assayed in multiple screens to confirm involvement in insulin or DAF-7 signaling.

Metabolic control by DAF-7 protein may be tested using any known cell line, e.g., those described herein.

### **In Vitro Screening Methods**

A variety of methods are also available for identifying useful compounds in *in vitro* assays. In one particular example, test compounds are screened for the ability to activate the phosphorylation of Smad proteins, DAF-8, DAF-14, or DAF-3, by DAF-1 or DAF-4 *in vitro*. In these assays, DAF-8, DAF-14, or DAF-3 is preferably tagged with a heterologous protein domain, for example, the myc epitope tag domain(s) by the method of Ausubel et al., and are incubated with the C-terminal kinase domain of DAF-1 or DAF-4. Phosphorylation of the Smad proteins is preferably detected by immunoprecipitation using antibodies specific to the tag, followed by scintillation counting. Test compounds may be screened in high throughput microtiter plate assays. A test compound that effectively stimulates the phosphorylation of DAF-8, DAF-14, or DAF-3 is considered useful

in the invention. Using these same general assays, compounds that activate the phosphorylation of DAF-16 by AKT or GSK-3 may also be identified.

In another working example, test compounds are screened for the ability to inhibit the *in vitro* association of DAF-16 and the Smad proteins DAF-3, or preferentially activates the association of DAF-16 with DAF-8 and DAF-14, DAF-8, or DAF-14, or to inhibit the association of DAF-3 and DAF-16 with DNA *in vitro*. These assays are carried out by any standard biochemical methods that test protein-protein binding or protein-DNA binding. In one particular example, to test for interactions between proteins, each protein is tagged with a different heterologous protein domain (as described above). Immunoprecipitations are carried out using an antibody to one tag, and an ELISA assay is carried out on the immunoprecipitation complex to test for the presence of the second tag. In addition, if interaction capability is enhanced by a DAF or AKT kinase, this protein is also preferably included in the reaction mixture. Similarly, to test for interactions of these proteins with DNA, antibodies to the tag are utilized in immunoprecipitations, and the presence of the DNA detected by the presence of the DNA label in the immunoprecipitation complex. A test compound that effectively inhibits the association between these proteins or between DAF-3 and DAF-16 with DNA or both is considered useful in the invention.

In still another working example, test derivatives of PIP3 are screened for the ability to increase *in vitro* AKT activity. This is accomplished, in general, by combining a labeled PIP3 and an AKT polypeptide in the presence and absence of the test compound under conditions that allow PIP3:AKT to bind *in vitro*. Compounds are then identified that interfere with the formation of the PIP3:AKT complex. Test compounds that pass this first screen may then be tested for increased AKT activation *in vitro* using GSK3 targets, or may be tested in nematodes or mammalian cells (as described above). An increase in AKT kinase



activity is taken as an indication of a compound useful for ameliorating or delaying an impaired glucose tolerance condition.

In yet another working example, DAF-3 or DAF-16 may be expressed in a yeast one-hybrid assay for transcriptional activation. Methods for such assays are described in Brent and Ptashne (*Cell* 43:729-736, 1985). A test compound that blocks the ability of DAF-3 or DAF-16 or both to activate (or repress) transcription in this system is considered useful in the invention.

In a final working example, compounds may be screened for their ability to inhibit an interaction between any of DAF-3, DAF-8, and DAF-14, or between DAF-3 and DAF-16. These *in vivo* assays may be carried out by any “two-hybrid” or “interaction trap” method (for example, by using the methods described by Vijaychander et al (*Biotechniques* 20: 564-568)).

#### Modulatory Compounds

Our experimental results facilitate the isolation of compounds useful in the treatment of impaired glucose tolerance diseases that are antagonists or agonists of the insulin or TGF- $\beta$  signaling pathways identified in *C. elegans* and described above. Exemplary methods for the isolation of such compounds now follow.

#### Antagonists

As discussed above, useful therapeutic compounds include those which down regulate the expression or activity of DAF-3 or DAF-16. To isolate such compounds, DAF-3 or DAF-16 expression is measured following the addition of candidate antagonist molecules to a culture medium of DAF-3 or DAF-16-expressing cells. Alternatively, the candidate antagonists may be directly administered to animals (for example, nematodes or mice) and used to screen for their effects on DAF-3 or DAF-16 expression.

DAF-3 or DAF-16 expression is measured, for example, by standard

Northern blot analysis (Ausubel et al., *supra*) using a DAF-3 or DAF-16 nucleic acid sequence (or fragment thereof) as a hybridization probe. The level of DAF-3 or DAF-16 expression in the presence of the candidate molecule is compared to the level measured for the same cells, in the same culture medium, or in a parallel set of test animals, but in the absence of the candidate molecule. Preferred modulators for anti-diabetic or anti-obesity purposes are those which cause a decrease in DAF-3 or DAF-16 expression.

Alternatively, the effect of candidate modulators on expression or activity may be measured at the level of DAF-3 or DAF-16 protein production using the same general approach in combination with standard immunological detection techniques, such as Western blotting or immunoprecipitation with a DAF-3 or DAF-16-specific antibody (for example, the DAF-3 or DAF-16 antibodies described herein). Again, useful anti-diabetic or anti-obesity therapeutic modulators are identified as those which produce a decrease in DAF-3 or DAF-16 polypeptide production. Antagonists may also affect DAF-3 or DAF-16 activity without any effect on expression level. For example, the identification of kinase cascades upstream of DAF-3 and DAF-16 (as described herein) suggest that the phosphorylation state of these polypeptides is correlated with activity. Phosphorylation state may be monitored by standard Western blotting using antibodies specific for phosphorylated amino acids. In addition, because DAF-3 and DAF-16 are transcription factors, reporter genes bearing operably linked DAF-3 or DAF-16 binding sites (for example, the myoII enhancer element) may be used to directly monitor the effects of antagonists on DAF-3 or DAF-16 gene activity.

Candidate modulators may be purified (or substantially purified) molecules or may be one component of a mixture of compounds (e.g., an extract or supernatant obtained from cells). In a mixed compound assay, DAF-3 or DAF-16 expression is tested against progressively smaller subsets of the candidate

compound pool (e.g., produced by standard purification techniques, e.g., HPLC or FPLC; Ausubel et al., *supra*) until a single compound or minimal compound mixture is demonstrated to modulate DAF-3 or DAF-16 expression.

Candidate DAF-3 or DAF-16 antagonists include peptide as well as non-peptide molecules (e.g., peptide or non-peptide molecules found, e.g., in a cell extract, mammalian serum, or growth medium on which mammalian cells have been cultured).

Antagonists found to be effective at the level of cellular DAF-3 or DAF-16 expression or activity may be confirmed as useful in animal models (for example, nematodes or mice). For example, the compound may ameliorate the glucose intolerance and diabetic symptoms of mouse models for Type II diabetes (e.g., a db mouse model), mouse models for Type I diabetes, or models of streptozocin-induced  $\beta$  cell destruction.

A molecule which promotes a decrease in DAF-3 or DAF-16 expression or DAF-3 or DAF-16 activity is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to decrease the level or activity of native, cellular DAF-3 or DAF-16 and thereby treat a glucose intolerance condition in an animal (for example, a human).

If desired, treatment with an antagonist of the invention may be combined with any other anti-diabetic or anti-obesity therapies.

### **Agonists**

Also as discussed above, useful therapeutic compounds are those which up regulate the expression or activity of DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT. To isolate such compounds, expression of these genes is measured following the addition of candidate agonist molecules to a culture medium of DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14,

AGE-1, or AKT-expressing cells. Alternatively, the candidate agonists may be directly administered to animals (for example, nematodes or mice) and used to screen for effects on DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT expression.

DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT-expression is measured, for example, by standard Northern blot analysis (Ausubel et al., *supra*) using all or a portion of one of these genes as a hybridization probe. The level of DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT expression in the presence of the candidate molecule is compared to the level measured for the same cells, in the same culture medium, or in a parallel set of test animals, but in the absence of the candidate molecule. Preferred modulators for anti-diabetic or anti-obesity purposes are those which cause an increase in DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT expression.

Alternatively, the effect of candidate modulators on expression may be measured at the level of DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT protein production using the same general approach in combination with standard immunological detection techniques, such as Western blotting or immunoprecipitation with an appropriate antibody. Again, the phosphorylation state of these polypeptides is indicative of DAF activity and may be measured on Western blots. Useful anti-diabetic or anti-obesity modulators are identified as those which produce an increase in DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT polypeptide production.

Candidate modulators may be purified (or substantially purified) molecules or may be one component of a mixture of compounds (e.g., an extract or supernatant obtained from cells). In a mixed compound assay, DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT expression is tested

against progressively smaller subsets of the candidate compound pool (e.g., produced by standard purification techniques, e.g., HPLC or FPLC; Ausubel et al., *supra*) until a single compound or minimal compound mixture is demonstrated to modulate DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT expression.

Alternatively, or in addition, candidate compounds may be screened for those which agonize native or recombinant DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT activities. In one particular example, DAF-1 and DAF-4 phosphorylation of DAF-8 and DAF-14, or AKT phosphorylation of DAF-16, may be activated by agonists.

Candidate DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT agonists include peptide as well as non-peptide molecules (e.g., peptide or non-peptide molecules found, e.g., in a cell extract, mammalian serum, or growth medium on which mammalian cells have been cultured).

Agonists found to be effective at the level of cellular DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT expression or activity may be confirmed as useful in animal models (for example, nematodes or mice).

A molecule which promotes an increase in DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT expression or DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT activities is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to increase the level or activity of these native, cellular genes and thereby treat a glucose intolerance condition.

If desired, treatment with an DAF-1, DAF-2, DAF-4, DAF-7, DAF-8, DAF-11, DAF-14, AGE-1, or AKT agonist may be combined with any other anti-diabetic or anti-obesity therapies.

### Animal Model Systems

Compounds identified as having activity in any of the above-described assays are subsequently screened in any number of available diabetic or obesity animal model systems, including, but not limited to ob (Coleman, *Dibetologia* 14: 141-148, 1978; Chua et al., *Science* 271: 994-996, 1996; Vaisse et al., *Nature Genet.* 14:95-100, 1996), db (Chen et al., *Cell* 84: 491-495, 1996), agouti mice, or fatty rats (Takaga et al. *Biochem. Biophys. Res. Comm.* 225: 75-83, 1996). Test compounds are administered to these animals according to standard methods. Additionally, test compounds may be tested in mice bearing knockout mutations in the insulin receptor, IGF-1 receptor (e.g., Liu et al., *Cell* 75:59-72, 1993), IR-related receptor, DAF-7 homolog, or any of the *daf* (FKHR, AFX) genes described herein. Compounds can also be tested using any standard mouse or rat model of Type I diabetes, e.g., a streptozin ablated pancreas model.

In one particular example, the invention involves the administration of DAF-7 or its homolog as a method for treating diabetes or obesity. Evaluation of the effectiveness of such a compound is accomplished using any standard animal model, for example, the animal diabetic model systems described above. Because these mouse diabetic models are also associated with obesity, they provide preferred models for human obesity associated Type II diabetes as well. Such diabetic or obese mice are administered *C. elegans* or human DAF-7 according to standard methods well known in the art. Treated and untreated controls are then monitored for the ability of the compound to ameliorate the symptoms of the disease, e.g., by monitoring blood glucose, ketoacidosis, and atherosclerosis. Normalization of blood glucose and insulin levels is taken as an indication that the compound is effective at treating a glucose intolerance condition.

### Therapy

Compounds of the invention, including but not limited to, DAF-7 and its homologs, and any antagonist or agonist therapeutic agent identified using any of the methods disclosed herein, may be administered with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such compositions to patients. Although intravenous administration is preferred, any appropriate route of administration may be employed, for example, parenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for antagonists or agonists of the invention include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

DAF polypeptides are administered at any appropriate concentration, for example, for DAF-7, at a concentration of around 10nM.

#### **Pedigree Analysis and Genetic Testing**

The discovery described herein that DAF polypeptides are involved in glucose metabolism enables assays for genetic testing to identify those individuals with predispositions toward the development of glucose intolerance conditions, such as diabetes or obesity, by determining the presence of a mutation found in a human gene having identity to any of the *C. elegans daf-1, daf-2, daf-3, daf-4, daf-7, daf-8, daf-11, daf-14, daf-16, age-1, or akt* genes. In one embodiment, the development of this testing method requires that the individual be a member of a family that has multiple affected and unaffected members carrying one mutation from the list of above-listed genes. Those skilled in the art will understand that a diabetic or obesity phenotype may be produced by *daf, age, or akt* mutations found on different chromosomes, and that low resolution genetic mapping of the diabetic condition in single family pedigrees will be sufficient to favor some *daf, age, or akt* genes over others as causing the glucose intolerance condition in a particular pedigree. In one particular example, mutations associated with glucose intolerance may be found in different genes in, for example, the DAF-7 signaling pathway in each pedigree. In addition, because mutations in a common pathway can show complex genetic interactions, multiple DAF mutations may segregate in single pedigrees. These mutations can behave recessively in some genetic backgrounds and dominantly in others.

Those skilled in the art further understand that, to determine disease linkage with a chromosomal marker, it may be necessary to evaluate the association of inheritance patterns of several different chromosomal markers (for example, from the collection of highly polymorphic mapped DNA allelic variants) in the genomic



DNAs of family members and of the clinically affected individuals. Methods commonly used in determining segregation patterns of human genetic diseases are well known in the art. In addition, methods are known in the art for determining whether individuals in a family are useful for providing information to determine co-segregation of an allele with a glucose intolerance trait.

Here, fragments of genomic DNA (e.g., RFLP fragments) are prepared from each of the available members of the family, and each distinctive DNA allelic variant of the polymorphic chromosome marker within the family is evaluated to determine which polymorphisms (i.e., chromosomal region) is linked with the glucose intolerance phenotype within a particular family. It is preferred that the parents of the marker individual be heterozygous for a DNA allelic variant so that the segregation pattern of the DNA allelic variant linked with the diabetic or obese phenotype in the marker can be recognized. The inheritance of the diabetic phenotype can be judged to be dominant or recessive, depending on the pattern of inheritance. Most diabetes is dominantly inherited, and therefore inbred pedigrees are generally not necessary in the etiology of the diabetic condition.

With respect to Type II diabetes, the highest rate of this kind of diabetes in the world is found in American Indians of the Pima tribe. Such families are useful for mapping one particular cause of diabetes, but, in general, diabetes is caused by mutations in a variety of genes, including *daf* genes. Thus, by testing for low resolution linkage to a candidate *daf*, *age*, or *akt* mutation, and then by sequencing the particular linked *daf* gene in affected and unaffected individuals, a particular *daf* mutation can be associated with a particular diabetic pedigree.

Human DAF homologues are mapped to chromosome regions using standard methods. For example, the probable DAF-16 homologue FKHR is located on chromosome 13, and AFX is located on the X chromosome. Any *daf*, *akt*, or *age* genes mapping to the approximate chromosomal regions associated

with diabetes or glucose intolerance are sequenced from affected and unaffected individuals. Preferably, at least two genes per pedigree of 5-20 affected (and unaffected controls) are sequenced. The *daf* genomic regions are PCR amplified and compared between affected and unaffected DNA samples. Mutations detected in affected individuals are expected to (but need not) map to conserved domains of the DAF genes. Because it is known that not all carriers of known diabetes-inducing mutations show metabolic defects, we expect that some non-diabetic non-glucose intolerant family members will carry the same *daf* mutation as affected family members. For this reason, a correlation of affected family members with a *daf* mutation is more important than a correlation of nonaffected with no mutation. Those skilled in the art will know that phenotypic classification of affected and unaffected individuals can greatly enhance the power of this genetic analysis (Nature Genet. 11: 241-247, 1995). In addition, other mutations in the same *daf* gene are expected in some but not all diabetic pedigrees. For dominant diabetic inheritance, the affected individuals carry a *daf*, *age*, or *akt* mutation as well as a normal allele. For recessive diabetic inheritance, individuals carry two *daf* mutations that may be identical or two independent mutations in the same gene. In addition, some diabetic individuals may carry mutations in more than one *daf*, *age*, or *akt* gene (so called non-allelic non-complementation).

It is routine in the art of genetic counseling to determine risk factors given the presence of a closely linked molecular genetic marker in the genomic DNA of the individual and when combined with the additional understanding provided by the pedigree of the individual in the family. For example, a risk factor may be calculated for an individual in an *age*, *akt*, or *daf* chromosome family in a manner similar to those described for assessing the risk of other commonly known genetic diseases that are known to run in families, e.g., Huntington's disease and cystic fibrosis.

Once mutations in *daf*, *akt*, or *age* genes are associated with diabetes in a pedigree analysis, diagnostic PCR sequencing of these *daf* genes can be used to diagnose glucose intolerant, prediabetic, diabetic, obesity, and atherosclerotic conditions. Preferably, the *daf*, *akt*, or *age* gene regions are PCR amplified from patients and mutations detected in the *daf* genes using standard DNA sequencing or oligonucleotide hybridization techniques. The use of such gene sequences or specific antibody probes to the products of these sequences provide valuable diagnostics, particularly in view of the likelihood there exist two classes of type II diabetics: those with defects in the TGF- $\beta$  signaling genes, and those with defects in insulin signaling genes. Such genetic tests will influence whether drugs that affect DAF-7 TGF- $\beta$  or DAF-2 insulin like signals are prescribed.

To carry out the above analysis (as well as the other screening, diagnostic, and therapeutic methods described herein), mammalian homologs corresponding to the *C. elegans* *daf-1*, *age-1*, *daf-4*, *daf-8*, and *daf-7* genes are isolated as described above for *daf-2*, *daf-3*, and *daf-16*. Again, standard hybridization or PCR cloning strategies are employed, preferably utilizing conserved DAF, AGE, or AKT motifs for probe design followed by comparison of less conserved sequences flanking these motifs. Exemplary motifs for these genes are as follows:

DAF-1 (139 amino acid motif) (SEQ ID NO: 13)

274 TSGSGMGPTTLHKLTIGGQIRLTGRVGSGRFGNVSRGDYRGEAVAVK  
VFNALDEPAFHKETEIFETRMLRHPNVLR YIGSDRVD TG FVTELWLVT EYH  
PSGSLHD FLENTVNIETYYNL MRSTASGLAFLHNQIGGSK 412

DAF-1 (62 amino acid motif) (SEQ ID NO: 14)

450 EDAASDIANENYKCGTVRYLAPEILNSTMQFTVFESYQCADVYSFSL  
VMWETLCRCEDGDV 511

DAF-1 (31 amino acid motif) (SEQ ID NO: 15)

416 KPAMAHARDIKSKNIMVKNDLTCAIGDLGLSL 466

DAF-1 (72 amino acid motif) (SEQ ID NO: 16)

520 IPYIEWTDRDPQDAQMFDVVCTRRLRPTENPLWKDHPMKHIMEIKT  
CWNGNPSARFTS YICRKRMDERQQ 591

AGE-1 (150 amino acid motif) (SEQ ID NO: 17)

991 YFESVDRFLYSCVGYSVATYIMGIKDRHSDNMLMLTEDGKYVHIDFGHI  
LGHGKTKLGIQRDRQPFILTEHFMTVIRSGKSVDGNSHELQKFKTLCVEAY  
EVMWNNRDLFVSLFTLMLGMELPELSTKADLDHLKKTLFCNGESKEEAR  
KF 1140

**AGE-1** (113 amino acid motif) (SEQ ID NO: 18)

826 SPLDPVYKLGEMIIDKAIVLGS AKRPLMLHWKNKNPKSDLHL PFCAMI  
FKNGDDL RQDMLVLQVLEVMDNIWKAANIDCCLNPYA VLP MGEMIGIIE  
VVPNCKTIFEIQVGTG 938

AGE-1 (106 amino acid motif) (SEQ ID NO: 19)

642 LAFVWTDRENFSELVVMLEKWKPPSVAAALTLGKRCTDRVIRKFAV  
EKLNEQLSPVTFHLFILPLIQALKYEPRAQSEVGMMLLTRALCDYRIGHRLF  
WLLRAEI 747

AGE-1 (60 amino acid motif) (SEQ ID NO: 38)

91 EIKLSDFKHQLFELIAPMKWGTYSVKPQDYVFRQLNNGEIEVIFND  
DQPLSKLELHGTF 150

AKT (121 amino acid motif) (SEQ ID NO: 60)

33685 QVLDDHDYGRCDWWGVGVVMYEMMCGRLPFYSKDHNKLF  
ELIMAGDLRFPSKLSQEARTLLTGLLVKDPTQRLGGGPEDALEICRADFFR  
TVDWEATYRKEIEPPYKPNVQSETDTSYFD 34047

AKT (66 amino acid motif) (SEQ ID NO: 61)

32314 TMEDFDLKVGLKGTFGKVILCKEKRTQKLYAIKILKKDVIIARE  
EVAHTLTENRVLQRCKHPFLT 32511

AKT (45 amino acid motif) (SEQ ID NO: 62)

33509 KLENLLLDKDGHIKIADFGLCKEEISFGDKTSTFCGTPEYL  
APEV 33643

AKT (57 amino acid motif) (SEQ ID NO: 63)

32667 YFQELKYSFQEQHYLCFVMQFANGGELFTHVRKCGTFSEPRARFY  
GAEIVLALGYLH 32837

AKT (59 amino acid motif) (SEQ ID NO: 64)

31846 STFAIFYFQTMLFEKPRPNMFMVRCLQWTTVIERTFYAESAEVRQ  
RWIHAIESISKKYK 32022

AKT (33 amino acid motif) (SEQ ID NO: 65)

33156 LQELKYSFQTNDRLCFVMEFAIGGDLYYHLNRE 33254

AKT (21 amino acid motif) (SEQ ID NO: 66)

30836 VVIEGWLHKKGEHIRNWRPRF 30898

AKT (26 amino acid motif) (SEQ ID NO: 67)

33276 FSEPRARFYGSEIVLALGYLHANSIV 33353

DAF-4 (139 amino acid motif) (SEQ ID NO: 20)

380 EYWIVTEFHERLSLYELLKNNVISITSANRIIMSMIDGLQFLHDDRPYFF  
GHPKKPIIHRDIKSKNILVKSDMTTCIADFGLARIYSYDIEQSDLLGQVGTK  
RYMSPEMLEGATEFTPTAFKAMDVYSMGLVMWEVISR 518

DAF-4 (61 amino acid motif) (SEQ ID NO: 21)

537 IGFDPTIGRMRNYVVSKKERPQWRDEIHKHEYMSLLKKVTEEMWDPE  
ACARITAGCAFARV 597

DAF-4 (20 amino acid motif) (SEQ ID NO: 22)

305 PITDFQLISKGRFGKVFKAQ 324

DAF-8 (163 amino acid motif) (SEQ ID NO: 23)

382 TDSETRSRFSLGWYNNPNRSPQTAEVRLIGKGVRFYLLAGEVYVENL  
CNIPVFVQSIGANMKNGFQLNTVSKLPPTGTMKVFDMLFSKQLRTAAEK  
TYQDVYCLSRMCTVRVSFCKGWGEHYRRSTVLRSPVWFQAHLNNPMHW  
VDSVLTCMGAPPRICSS 544

DAF-8 (44 amino acid motif) (SEQ ID NO: 24)

91 RAFRFPVIRYESQVKSILTCRHAFNSHSRNVCLNPYHYRWVELP 134

DAF-8 (38 amino acid motif) (SEQ ID NO: 25)

341 VEYEEPSWLKLIYYEETMIGEKAADVEGHHCLIDGFT 378

DAF-14 (39 amino acid motif) (SEQ ID NO: 68)

9709 IRVSFCKGFGETY SRLKVVNLPCWIEIILHEPADEYDTV 9825

DAF-14 (45 amino acid motif) (SEQ ID NO: 69)

9409 SRNSKSSQIRNTVGAGIQLAYENGELWLTVLTDQIVFVQCPFLNQ  
9543

DAF-14 (29 amino acid motif) (SEQ ID NO: 70)

9160 NEMLDPEPKYPKEEKPWCTIFYYELTVRV 9246

DAF-14 (29 amino acid motif) (SEQ ID NO: 71)

9307 QLGKAFAKVPTITIDGATGASDECRMSL 9393

DAF-12 (105 amino acid motif) (SEQ ID NO: 72)

103 SPDDGLLDSSSEESRRRQKTCRVCGDHATGYNFNVITCESCKAFFRR  
NALRPKEFKCPYSEDCEINSVSRRFCQKCRLRKCF TVGMKKEWILNEEQ LR  
RRKNSRLN 207

DAF-12 (89 amino acid motif) (SEQ ID NO: 73)

109 LDSSEESRRRQKTCRVCGDHATGYNFNVITCESCKAFFRRNALRPKE  
FKCPYSEDCEINSVSRRFCQKCRLRKCF TVGMKKEWILNEEQ 197

DAF-12 (73 amino acid motif) (SEQ ID NO: 74)

551 DIMNIMDVTMRRFVKVAKGVPAFREVSQEGKFSLLKGGMIEMLT  
V RGVTRYDASTNSFKTPTIKGQNVSVNVD 623

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DAF-11 (112 amino acid motif) (SEQ ID NO: 75)

708 SGSLVDLMIKNLTAYTQGLNETVKNRTAELEKEQEKGQDQLLMELL  
PKSVANDLKNGIAVDPKVYENATILYSDIVGFTSLCSQSQPMEEVVTLLSGM  
YQRFDLIISQQGGYKV 819

DAF-11 (107 amino acid motif) (SEQ ID NO: 76)

825 METIGDAYCVAAGLPVVMKDHVKSICMIALLRDCLHHFEIPHR  
PGTFLNCRWGFNSGPVFAGVIGQKAPRYACFGEAVILASKMESSGVEDRIQ  
MTLASQQLLEE 931

DAF-11 (43 amino acid motif) (SEQ ID NO: 77)

520 DILKGLEIYHASAIDFHGNLTLCMLDSHWIVKLSGFGVNRL 562

DAF-11 (15 amino acid motif) (SEQ ID NO: 78)

618 DMYSFGVILHEILK 632

DAF-7 (60 amino acid motif) (SEQ ID NO: 26)

290 NLAETGHSKIMRAAHKVSNP EIGYCCHPTEYDYIKLIYVNRDGRVSIA  
NVNGMIAKKCGC 349

DAF-7 (20 amino acid motif) (SEQ ID NO: 27)

265 DWIVAPPRYNAYMCRGDCHY 284

DAF-7 (43 amino acid motif) (SEQ ID NO: 28)

240 VCNAEAQSKGCCLYDLEIEFEKIGWDWIVAPPRYNAYMCRGDC 282



DAF-7 (70 amino acid motif) (SEQ ID NO: 29)

281 DCHYNAHHFNLAETGHSKIMRAAHKVSNP EIGYCCHPTEYDYIKLIYV  
NRDGRVSIANVN GMIAKKCGCS 350

DAF-7 (35 amino acid motif) (SEQ ID NO: 30)

250 CCLYDLEIEFEKIGWDWIVAPPRYNAYMCRGDCHY 284

DAF-7 (13 amino acid motif)(SEQ ID NO: 51)

GWDWIVAPPRYNA

In one particular example, mammalian DAF-7 may be identified using the sub-domain amino acids 314-323. Exemplary degenerate oligonucleotides designed to PCR amplify this domain or hybridize (for example, as described in Burglin et al., (Nature 341:239-243, 1989) are as follows:

aa 263 oligo: GGNTGGGAYTRNRTNRTNGCNCC (23-mer, 16,000-fold degeneracy) (SEQ ID NO: 31)

aa 314 oligo: TGYTGYNNNCCNACNGAR (18-mer, 8000-fold degeneracy) (SEQ ID NO: 32).

The DNA sequence between the oligonucleotide probes is determined, and those sequences having the highest degree of homology are selected. Once isolated, these sequences are then tested in a *C. elegans daf-7* mutant or mouse model as described above for the ability to functionally complement the mutation or ameliorate the glucose intolerance phenotype.

### Other Embodiments

In other embodiments, the invention includes any protein which possesses the requisite level of amino acid sequence identity (as defined herein) to DAF-2, DAF-3, or a DAF-16 sequence; such homologs include other substantially pure naturally-occurring mammalian DAF polypeptides (for example, human DAF polypeptides) as well as allelic variants; natural mutants; induced mutants; proteins encoded by DNA that hybridizes to the DAF DNA sequence or degenerate conserved domains of DAF proteins (e.g., those described herein) under high stringency conditions; and proteins specifically bound by antisera directed to a DAF-2, DAF-3, or DAF-16 polypeptide.

The invention further includes analogs of any naturally-occurring DAF-2, DAF-3, or DAF-16 polypeptides. Analogs can differ from the naturally-occurring protein by amino acid sequence differences which do not destroy function, by post-translational modifications, or by both. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring DAF polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989, or Ausubel et al., *supra*). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g.,  $\beta$  or  $\gamma$  amino acids.

In addition to full-length polypeptides, the invention also includes DAF-2, DAF-3, and DAF-16 polypeptide fragments. As used herein, the term "fragment," means at least 20 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of such DAF polypeptides can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

For certain purposes, all or a portion of the DAF-2, DAF-3, or DAF-16 polypeptide sequence may be fused to another protein (for example, by recombinant means). In one example, the DAF polypeptide may be fused to the green fluorescent protein, GFP (Chalfie et al., *Science* 263:802-805, 1994). Such a fusion protein is useful, for example, for monitoring the expression level of the DAF polypeptide *in vivo* (for example, by fluorescence microscopy) following treatment with candidate or known DAF agonists or antagonists.

The methods of the invention may be used to diagnose or treat any condition related to glucose intolerance or obesity in any mammal, for example, humans, domestic pets, or livestock. Where a non-human mammal is diagnosed or treated, the DAF polypeptide, nucleic acid, or antibody employed is preferably specific for that species.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

Other embodiments are within the following claims.



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